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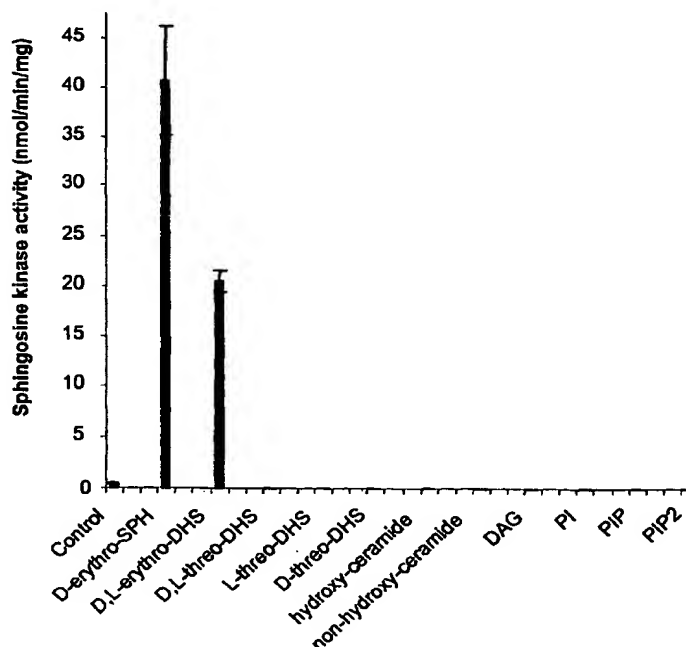
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(54) Title: HUMAN SPHINGOSINE KINASE GENE



(57) Abstract: The present invention relates to the human sphingosine kinase type 1 gene. More precisely the invention concerns a purified or isolated nucleic acid of said sphingosine kinase or a sequence complementary thereto, or fragments thereof. The invention includes oligonucleotides, recombinant polypeptides, recombinant vectors, recombinant host cells comprising said nucleic acid, as well as antibody production, methods of screening, antisense oligonucleotide, knock out mammals.

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HUMAN SPHINGOSINE KINASE GENE

FIELD OF THE INVENTION

5 The present invention relates to the human sphingosine kinase type 1 gene. More precisely the invention concerns a purified or isolated nucleic acid of said sphingosine kinase or a sequence complementary thereto, or fragments thereof. The invention includes oligonucleotides, recombinant polypeptides, recombinant vectors, recombinant host cells comprising said nucleic acid, as well as antibody
10 production, methods of screening, antisense oligonucleotide, knock out mammals.

BACKGROUND OF THE INVENTION

15 Sphingosine-1-phosphate, the product of sphingosine kinase, is an important signaling molecule with intra- and extracellular functions. The cDNA for the mouse sphingosine kinase has recently been reported as described in patent application number WO 99/61581. The mouse SK1A and SK1B are presumably alternative splice forms. Differential splicing probably results in two variants of
20 the N-terminal peptide sequence and it is the consequence of alternative coding exon usage (Kohama et al., 1998).

SUMMARY OF THE INVENTION

25 The invention concerns a purified or isolated nucleic acid encoding a human sphingosine kinase (hereinafter hSK) cDNA or a sequence complementary thereto.

Oligonucleotide probes or primers specifically hybridizing to a nucleic acid encoding hSK, to fragments thereof or to a sequence complementary thereto are
30 also part of the invention as well as DNA amplification and detection methods using said primers and probes.

CONFIRMATION COPY

A further object of the present invention concerns recombinant vectors comprising any of the nucleic acid sequences described herein, and in particular recombinant vectors comprising a nucleic acid sequence encoding a recombinant hSK.

The invention also includes recombinant expression vectors comprising a nucleic acid sequence encoding recombinant hSK.

The invention also encompasses host cells and transgenic non-human mammals comprising said nucleic acid sequences or recombinant vectors.

The invention further concerns an isolated recombinant hSK.

The invention also concerns a hSK polypeptide or a peptide fragment thereof as well as antibodies specifically directed against a peptide of hSK.

The invention further concerns a method for the screening of candidate molecules which are inhibitors of hSK.

The method comprises the steps of:

- mixing a recombinant hSK with sphingosine, labelled ATP and a candidate molecule of interest; and
- measuring the level of conversion of sphingosine to labelled-sphingosine-1-phosphate (S1P).

The invention also concerns a kit for the screening of candidate molecules which are inhibitors of hSK.

The kit comprises:

- recombinant hSK; and, optionally,
- labelled ATP and sphingosine.

The invention also concerns inhibitors of hSK1 obtained through the screening method described above, structural analogues thereof, and their use in the treatment or prevention of one and/or several disease states selected from: degenerative disease processes such as atherosclerosis and fibrosis; neurodegenerative disorders; cardiovascular diseases including atherosclerosis, thrombosis and dyslipidemia; diabetes including type I and type II diabetes and particularly type I diabetes; stroke; autoimmune and inflammatory diseases such as multiple sclerosis, psoriasis, epidermodysplasia verruciformis and inflammatory arthritis; T helper-1 related diseases; chronic obstructive pulmonary disease; asthma; cancer; hemostasis, stroke, coronary artery disease, hematopoietic disorders such as leukemia, the natural wound healing processes, myocardial infarction, embryogenesis.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates the cDNA and predicted amino acid sequence of a human sphingosine kinase 1.

Figure 2A and 2B shows respectively the predicted secondary structure and the conserved regions of human sphingosine kinase type 1.

Figure 3 illustrates hSK1 substrate recognition

Figures 4 A (4A1, 4A2) and 4B (4B1, 4B2) show that hSK1 has high specificity for *D-erythro*-sphingosine and illustrate that hSK1 is inhibited by *D,L*-threo-dihydrosphingosine and *N,N*,diMethyl-sphingosine.

Figure 5 A describes the expression and cellular localisation of hSK1 fused with EGPF at the N-terminal end.

Figure 5B illustrates the expression and cellular localisation of hSK1 fused with EGPF at the C-terminal end.

Figure 6 shows the kinase activity of hSK fusion proteins.

Figure 7 describes the expression levels of hSK fusion proteins.

Figure 8 shows the tissue distribution of hSK1 messenger RNA.

Figure 9 illustrates the comparison of hSK activity from different sources: CHO cells, Bacteria, partially purified hSK1 from insect cells.

Figure 10 illustrates the comparison of hSK1 activity from different sources: Cos7, bacteria, insect cells.

Figure 11 describes the bacterial growth conditions for optimization of actively expressed hSK1.

Figure 12 shows the comparison of hSK1 activity expressed under different bacterial growth conditions and expressed in Cos cells.

The hSK1 activity under optimal bacterial growth and induction conditions (50 μ M IPTG for 20hr) is 40% of the activity observed for the transfected Cos7 cells extract.

Figure 13 illustrates the physiological relevant role of hSK1 proven by the use of an antisense oligonucleotide.

Figure 14 shows the vector for the construction of hSK-EGFP (N-terminal) fusion for expression in mammalian cells.

Figure 15 illustrates the vector for the construction of hSK-EGFP(C-terminal fusion) for expression in mammalian cells.

Figure 16 illustrates the vector for the construction of hSK1 tagged with GST for expression in bacterial cells.

Figure 17 shows an electrophoresis gel of the partial purification of hSK1 from Sf21 insect cells.

Figure 18 illustrates the antisense downregulation of hSK1 protein levels.

DETAILED DESCRIPTION OF THE INVENTION

A) Human sphingosine kinase cDNA

A first object of the present invention is a purified or isolated nucleic acid encoding hSK, or a sequence complementary thereto.

Another object of the invention is a purified or isolated nucleic acid having at least 90%, preferably 95%, more preferably 98% and most preferably 99% nucleotide identity with the nucleotide sequence of SEQ ID N°1 or of SEQ ID N°2, or a sequence complementary thereto.

A further object of the present invention is a purified or isolated nucleic acid encoding a polypeptide having at least 80%, preferably 90%, more preferably 95%, and most preferably 98 or 99% amino-acid identity with the human polypeptide of the amino-acid sequence of SEQ ID N°3 or with a peptide fragment thereof, or a sequence complementary thereto.

Polypeptides having amino-acid identity with the hSK of the invention encompass polypeptides:

- that have primary structures which are related to the hSK of the amino-acid sequence of SEQ ID N°3, due to the high sequence identity between the amino-acid sequences; or

-that are biologically related to the polypeptides of the amino-acid sequence of SEQ ID N°3, either because these homologous polypeptides are recognized by antibodies specifically directed against the amino-acid sequence of SEQ ID N°3 and/or because these homologous polypeptides have the same biological activity as the polypeptides of the amino-acid sequence of SEQ ID N°3, such as for example the capacity to convert sphingosine into S1P.

The term "isolated", when used herein, requires that the material be removed from its original environment (e.g. the natural environment if it is naturally occurring). For example, a naturally occurring polynucleotide or a peptide present in a living animal is not isolated, but the same polynucleotide or peptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotide can be part of a vector and/or such polynucleotide or peptide can be part of a composition, and still be isolated. This is so because the vector or composition is not part of the original environment of the nucleotide sequence it contains.

The term "purified" does not require absolute purity; rather, it is intended as a relative definition. Purification of starting materials or natural materials to at least one order of magnitude, preferably two or three orders, and more preferably four or five orders of magnitude is expressly contemplated.

Throughout the present specification, the expression "nucleotide sequence" is used to designate indifferently a polynucleotide or a nucleic acid. More precisely, the expression "nucleotide sequence" encompasses the nucleic material and the sequence information and is not restricted to the sequence information (i.e. the succession of letters chosen among the four base letters) that biochemically characterizes a specific DNA or RNA molecule.

As used interchangeably herein, the terms "oligonucleotides", "nucleic acids" and "polynucleotides" include RNA, any type of DNA such as genomic DNA, cDNA or RNA/DNA hybrid sequences of more than one nucleotide in either single chain or duplex form.

Further to its general meaning understood by one skilled in the art, the term "nucleotide" is also used herein to encompass modified nucleotides which comprise at least one of the following modifications:

- (a) an alternative linking group,
- (b) an analogous form of purine,
- (c) an analogous form of pyrimidine, or
- (d) an analogous sugar;
- (e) modified nucleotides such as methylated, phosphorylated, ubiquitinated nucleotides.

For examples of analogous linking groups, purines, pyrimidines, and sugars, see for example PCT publication N°WO 95/04064.

The polynucleotide sequences of the invention may be prepared by any known method, including synthetic, recombinant, or a combination thereof as well as through any purification methods known in the art.

B) Recombinant hSK polynucleotides

The invention also encompasses polynucleotide fragments of a nucleic acid encoding the hSK1 of the invention. These fragments particularly include but are not restricted to 1) those fragments encoding a polypeptide of hSK which preferably retains its affinity for sphingosine and 2) nucleotide fragments useful as nucleic acid primers or probes for amplification or detection purposes.

A most preferred embodiment of this invention for a fragment encoding a polypeptide of hSK is the polynucleotide of sequence SEQ ID NO: 8 corresponding to a region of SK conserved between species. In fact the inventors have shown that a 80 amino-acids long region of hSK1 is conserved between species (figure8).

- **Primers or probes**

More particularly, the present invention concerns a purified or isolated polynucleotide comprising at least 10 consecutive nucleotides of a nucleic acid encoding the hSK described herein, preferably at least 10 consecutive nucleotides of the nucleotide sequence of SEQ ID N°1 or of SEQ ID N°2, or a sequence complementary thereto.

These nucleic acids consist of a contiguous span which ranges in length from 10, 12, 15, 18 or 20 to 25, 35, 40, 50, 70, 80, 100, 250, 500 or 1000 nucleotides, or be specified as being 10, 12, 15, 18, 20, 25, 35, 40, 50, 100, 200, 250, 500 or 1000 nucleotides in length.

In one particular embodiment of this invention these nucleic acids are useful as probes in order to detect the presence of at least a copy of a nucleotide sequence encoding hSK, more particularly the presence of at least a copy of a nucleotide sequence of SEQ ID N°1 or of SEQ ID N°2 or a sequence complementary thereto or a fragment or a variant thereof in a sample. The sequence of such nucleic acids could be slightly modified (for example by substituting one nucleotide for another) without substantially affecting the ability of such modified sequence to hybridize with the targeted sequence of interest.

The most preferred probes are the following:

SK5'end49 (gene proximal) CTGGGTCTTGTAGAAGAGCAGCAAGTGCT
(SEQ ID NO: 14)

SK5'end48 (gene proximal)

AGTTCACCTGCAATCCTTTCTTATCTGGGTTCG (SEQ ID NO: 15)

SK3'end (gene distal) TTCTGTGGATGGAGAGCTGATGGTATGG (SEQ
ID NO: 16)

SK BOX (conserved region) ATGAAGTGGTGAATGGGCTAATGGAACG
(SEQ ID NO: 17)

The nucleic acid probes of the invention may also be used for the analysis of the expression levels and patterns of hSK, such as described in the PCT Application

N^oWO 97/05 277, the entire contents of which is herein incorporated by reference.

In another embodiment of the invention these nucleic acids are useful as primers.

The most preferred primers are the following:

A= 5'end TAT GCT AGC ATG GAT CCA GCG GGC GGC (SEQ ID NO: 4)

B= 3'end AAT GAA TTC TCA TAA GGG CTC TTC TGG (SEQ ID NO: 5)

C= 5'end TTA GAA TTC CAC CAT GGA TCC AGC GGG CGG C (SEQ ID NO: 6)

D= 3'end ATT ATC GTC GAC TAA GGG CTC TTC TGG CGG (SEQ ID NO: 7)

E= 5'end TTA GAA TTC CAC CAT GGA TCC AGC GGG CGG C (SEQ ID NO: 10)

F= 3'end AGT CGA GGC TGA TCA GCG AG (SEQ ID NO: 11)

• Hybridizing polynucleotides

The invention also concerns purified or isolated nucleic acid sequences that hybridize, under stringent hybridization conditions, with a polynucleotide encoding hSK or a sequence complementary thereto.

A preferred embodiment of the invention is a purified or isolated nucleic acid sequence that hybridize, under stringent conditions, with the nucleic acid of 270 nucleotides (SEQ ID NO: 22) encoding the 80 amino acids conserved region of hSK1.

As an illustrative embodiment, stringent hybridization conditions can be defined as follows:

The hybridization step is conducted at 65°C in the presence of 6 x SSC buffer, 5 x Denhardt's solution, 0.5 % SDS and 100µg/ml of salmon sperm DNA.

The hybridization step is followed by four washing steps:

- two washings during 5 minutes, preferably at 65°C in a 2 x SSC and 0.1% SDS buffer;

- one washing during 30 minutes, preferably at 65°C in a 2 x SSC and 0.1% SDS buffer;

- one washing during 10 minutes, preferably at 35°C in a 0.1 x SSC and 0.1% SDS buffer,

It being understood that the hybridization conditions defined above are suitable for nucleic acids of approximately twenty nucleotides in length and that these conditions may be also adapted for shorter or longer nucleic acids, according to techniques well known in the art, for example those described by Sambrook et al. (1989).

The appropriate length for probes under a particular set of assay conditions may be empirically determined by the one skilled in the art. The probes can be prepared by any suitable method, including, for example, cloning and restriction of appropriate sequences and direct chemical synthesis by a method such as the phosphodiester method of Narang et al. (1979), the phosphodiester method of Brown et al., (1979), the diethylphosphoramidite method of Beaucage et al. (1981) and the solid support method described in the application N°EP-0 707 792. The disclosures of all these documents are incorporated herein by reference.

Any of the nucleic acids of the present invention can be labelled, if desired, by incorporating a label detectable by spectroscopic, photochemical, biochemical, autoradiographic, radiochemical, immunochemical, or chemical means. For example, useful labels include radio-active substances (^{32}P , ^{35}S , ^3H , ^{125}I),

fluorescent dyes (5-bromodesoxyuridin, fluorecein, acetylaminofluoren, digoxigenin) or biotin. Examples of non-radioactive labelling of nucleic acid fragments are described in French Patent N°FR-78 10975 or by Urdea et al. (1988) or Sanchez-Pescador et al. (1988).

5

Advantageously, the probes according to the present invention may have structures and characteristics such that they allow signal amplification, such structural characteristics being, for example, those of branched DNA probes as described by Urdea et al. (1991).

10

Any of the nucleic acid probes of the invention can be conveniently immobilized on a solid support. Solid supports are known those skilled in the art and include the walls of wells of a reaction tray, test tubes, polystyrene beads, magnetic beads, nitro-cellulose strips, membranes, microparticules such as latex particles, sheep red blood cells, duracytes and others.

15

The nucleic acids of the invention and particularly the nucleotide probes described above can thus be attached to or immobilized on a solid support individually or in groups of at least 2, 5, 8, 10, 12, 15, 20 or 25 distinct nucleic acids of the invention to a single solid support.

20

In a specific embodiment of a support on which nucleic acid probes of the invention are immobilized, such a support may also contain other immobilized probes, preferably probes that hybridize specifically with a nucleic acid encoding hSK, or a variant thereof, or a sequence complementary thereto, more preferably probes that hybridize specifically with the nucleic acid of 240 nucleotides (SEQ ID NO: 22) encoding the 80 amino acids conserved region of hSK1.

25

C) Amplification of the hSK cDNA

Another object of the invention consists of a method for the amplification of a nucleic acid encoding a hSK, said method comprising the steps of:

30

(a) mixing a test sample suspected of containing the target hSK nucleic acid, a fragment or a variant thereof, or a sequence complementary thereto, with an amplification reaction reagent comprising a pair of amplification primers as disclosed herein which can hybridize under stringent conditions, the hSK nucleic acid region to be amplified, and

(b) optionally, detecting the amplification products.

In a first preferred embodiment of the above method, the nucleic acid encodes a hSK polypeptide of SEQ ID N°3.

In a second preferred embodiment of the above amplification method, the amplification product is detected by hybridization with a labelled probe having a sequence which is complementary to the amplified region.

The invention also concerns a kit for the amplification of a nucleic acid encoding hSK, a fragment or a variant thereof, or a complementary sequence thereto in a test sample, wherein said kit comprises:

(a) a pair of oligonucleotide primers as disclosed in the present invention which can hybridize, under stringent conditions to the hSK nucleic acid to be amplified;

(b) optionally, the reagents necessary for performing the amplification reaction.

In a first preferred embodiment of the kit described above, the nucleic acid to be amplified encodes hSK polypeptide of SEQ ID N°3.

In a second preferred embodiment of the above amplification kit, the amplification product is detected by hybridization with a labelled probe having a sequence which is complementary to the amplified region.

D) Recombinant vectors and hosts cells for the expression of a recombinant hSK

1) Recombinant vectors

The present invention also encompasses a family of recombinant vectors comprising any one of the nucleic acids described herein. Firstly, the invention deals with a recombinant vector comprising a nucleic acid selected from the group consisting of:

(a) a purified or isolated nucleic acid encoding hSK polypeptide, and more preferably a polypeptide having at least 80% amino acid identity with the polypeptide of SEQ ID N°3, or a sequence complementary thereto; or

(b) a purified or isolated polynucleotide comprising at least 10 consecutive nucleotides of a nucleic acid described in (a) or a sequence complementary thereto.

In a first preferred embodiment a recombinant vector of the invention is used to introduce the inserted polynucleotide derived from the nucleic acid encoding hSK polypeptide in a suitable host cell, this polynucleotide being amplified every time the recombinant vector replicates.

Recombinant expression vectors comprising a nucleic acid encoding hSK polypeptides that are described in the present specification are also part of the invention.

Another preferred embodiment of the recombinant vectors according to the invention consist of expression vectors comprising a nucleic acid encoding a hSK polypeptide of the invention, and more preferably a nucleic acid encoding a polypeptide having the amino acid sequence of SEQ ID N°3.

Preferred vectors comprises a nucleic acid sequence as shown in SEQ ID N° 1 or SEQ ID N°2.

Within certain embodiments, expression vectors can be employed to express a recombinant hSK polypeptide which can then be purified and for example, be used as an immunogen in order to raise specific antibodies.

Preferred eukaryotic vectors of the invention are listed hereafter as illustrative but not limitative examples: pcDNA3, pFLAG, pCMV-Script, pIND, pMC1NEO, pHIL, pGAPZA, pMT/V5-His-TOPO, pMT/V5-His, pAc5.1/V5-HisA, pDS47/V5-His, pcDNA4, pcDNA6, pEF1, pEF4, pEF6, pUB6, pZeoSV2, pRc/CMv2, pcDM8, pCR3.1, pDisplay, pSecTag2, pVP22, pEMZ, pCMV/Zeo, pSinRep5, pCEP, pREP, pHook-1.

The vectors pcDNA3, pFLAG, and pCMV (particularly pCMV5) are most preferred.

Preferred bacteriophage recombinant vectors of the invention are P1 bacteriophage vectors such as described by Sternberg N.L. (1992;1994).

A suitable vector for the expression of a recombinant hSK is a baculovirus vector that can be propagated in insect cells and in insect cell-lines such as Sf9 and Sf21. Specific suitable host vectors includes, but are not restricted to pFastBac-1, pIZ/V5-His, pBacMan-1, pBlueBac4.5, pBlueBacHis2, pMelBacA, pVL1392, pVL1393

Preferred baculovirus vector is pFastBacHTa.

A preferred bacterial vector is pGEX.

a) Regulatory expression sequences

Expression requires that appropriate signals are provided in the vectors, said signals including various regulatory elements such as enhancers/promoters from both viral and mammalian sources that drive expression of the genes of interest in host cells. The regulatory sequences of the expression vectors of the invention are operably linked to the nucleic acid encoding the recombinant hSK.

As used herein, the term "operably linked" refers to a linkage of polynucleotide elements in a functional relationship. For instance, a promoter or an enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence.

More precisely, two DNA molecules (such as a polynucleotide containing a promoter region and a polynucleotide encoding a desired polypeptide or polynucleotide) are said to be "operably linked" if the nature of the linkage between the two polynucleotides does not: (1) result in the introduction of a frame-shift mutation or (2) interfere with the ability of the polynucleotide containing the promoter to direct the transcription of the coding polynucleotide.

Generally, recombinant expression vectors include origins of replication, selectable markers permitting transformation of the host cell, and a promoter derived from a highly expressed gene to direct transcription of a downstream structural sequence. The heterologous structural sequence is assembled in an appropriate frame with the translation, initiation and termination sequences, and preferably a leader sequence capable of directing sequences of the translated protein into the periplasmic space or the extra-cellular medium.

In a specific embodiment wherein the vector is adapted for transfecting and expressing desired sequences in eukaryotic host cells, preferred vectors comprise an origin of replication from the desired host, a suitable promoter and an enhancer, and also any necessary ribosome binding sites, polyadenylation site, transcriptional termination sequences, and optionally 5'-flanking non-transcribed sequences.

DNA sequences derived from the SV 40 viral genome, for example SV 40 origin early promoter, enhancer, and polyadenylation sites may be used to provide the required non-transcribed genetic elements, another suitable promoter is the CMV promoter.

b) Promoter sequences

Suitable promoter regions used in the expression vectors according to the invention are chosen taking into account the host cell in which the heterologous nucleic acids have to be expressed.

A suitable promoter may be heterologous with respect to the nucleic acid for which it controls the expression, or alternatively can be endogenous to the native polynucleotide containing the coding sequence to be expressed.

Additionally, the promoter is generally heterologous with respect to the recombinant vector sequences within which the construct promoter/coding sequence has been inserted.

2) Recombinant host cells

Host cells that have been transformed or transfected with one of the nucleic acids described herein, or with one of the recombinant vector, particularly recombinant expression vector, described herein are also part of the present invention.

Are included host cells that are transformed (prokaryotic cells) or are transfected (eukaryotic cells) with a recombinant vector such as one of those described above. Preferred host cells used as recipients for the expression vectors of the invention are the following:

(1) prokaryotic host cells: bacterial cells and more particularly *Escherichia coli*, strains. (i.e. BL21, DH10 Bac strain) *Bacillus subtilis*, *Salmonella typhimurium* and strains from species such as *Pseudomonas*, *Streptomyces* and *Staphylococcus*; Sf-9 cells (ATCC N°CRL 1711), Sf 21 cells.

(2) eukaryotic host cells: HeLa cells (ATCC N°CCL2; N°CCL2.1; N°CCL2.2), Cv 1 cells (ATCC N°CCL70), COS cells (ATCC N°CRL 1650; N°CRL 1651), C127 cells (ATCC N°CRL-1804), 3T3 cells (ATCC N°CRL-6361), CHO cells (ATCC N°CCL-61), human kidney 293 cells (ATCC N°45504; N°CRL-1573), BHK (ECACC N°84100 501; N°84111301) and hi-5 cells.

More particularly, expressions of the recombinant hSK of the invention in COS-7 or in bacterial cells are preferred embodiment of the invention.

The results reported in the examples show that expression in Cos 7 and in bacteria are suitable for the production of an important amount of sphingosine kinase.

5 **E) Production of recombinant hSK**

The present invention also concerns a method for producing one of the amino acid sequences described herein and especially the polypeptide having the amino acid sequence of SEQ ID N°3, wherein said method comprises the steps of:

- 10 (a) inserting the nucleic acid encoding the desired amino acid sequence in an appropriate vector; or in a host cell;
- (b) culturing, in an appropriate culture medium, a host cell previously transformed or transfected with the recombinant vector of step (a);
- (c) harvesting the culture medium thus obtained or lyse the host cell, for
15 example by sonication or osmotic shock;
- (d) separating or purifying, from said culture medium, or from the pellet of the resultant host cell lysate, the thus produced recombinant polypeptide of interest.

20 In some instances, it may be required to tag the recombinant hSK prior to purification. The tag is then in most instances encoded into the nucleotide sequence that is needed to express the polypeptide. Examples of such tags include, but are not limited to sequences encoding C-myc, FLAG, a sequence of histidine residues, heamagglutinin A, V5, Xpress or GST. Most of these tags can be
25 incorporated directly into the sequence, for instance through PCR amplification by incorporating the appropriate coding sequence in one of the PCR amplification primers.

30 One preferred tag is the FLAG octapeptide (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys, SEQ ID NO: 23) which is used to express the recombinant hSK of the invention as a fusion protein. Both amino-terminal and carboxy-terminal FLAG

fusion proteins fall within the scope of the present invention. In preferred embodiments, the FLAG fusion proteins are produced through vectors which are derivatives of the pCMV-5 vector. More particularly, a pFLAG-CMV-1 or pFLAG-CMV-2 vector can be used for amino-terminal tagging whereas a pFLAG-CMV-5a, -5b or 5c vector can be used for carboxy-terminal tagging.

However, the tag can also be introduced by other means such as covalent binding of the appropriate nucleic acid sequence encoding the tag moiety with the 3' or 5' end of the nucleic acid sequence encoding the polypeptide sequence. This is the case for GST.

Purification of the recombinant hSK according to the present invention is then carried out by passage onto a nickel or copper affinity chromatography column, such as a Ni NTA column.

In another embodiment of the above method, the polypeptide thus produced is further characterized, for example by binding onto an immuno-affinity chromatography column on which polyclonal or monoclonal antibodies directed to the hSK of interest have been previously immobilised.

According to the results the production rate is higher for bacterial expression than for insect cells expression.

F) Purified recombinant hSK

Another object of the present invention consists of a purified or isolated recombinant polypeptide comprising the amino acid sequence of hSK.

Preferred isolated recombinant polypeptides of the invention include those having at least 80%, preferably 90%, more preferably 95, and most preferably 98

or 99%, amino-acid identity with the polypeptide having the amino acid sequence of SEQ ID N°3.

Extract of infected insect cells expressing a tagged hSK1 may be purified through resin column having affinity for the tag.

In a particular embodiment, extract of infected insect cells expressing a 6His tagged hSK1 are run through NI-NTA resin column.

In another embodiment, extract of infected insect cells expressing a GST tagged hSK1 are purified through glutathion resin.

G) Modified recombinant hSK

The invention also relates to a recombinant hSK polypeptide comprising amino acid changes ranging from 1, 2, 3, 4, 5, 10, 20, 25, 30, 35, 40 substitutions, additions or deletions of one amino acid as regards to polypeptides of anyone of the amino acid sequences of the present invention. Preferred sequences are those of SEQ ID N°3.

Amino acid changes encompassed are those which will not abolish the biological activity of the resulting modified polypeptide. These equivalent amino-acids may be determined either by their structural homology with the initial amino-acids to be replaced, by the similarity of their net charge or of their hydrophobicity, and optionally by the results of the cross-immunogenicity between the parent peptides and their modified counterparts.

Alternatively, in the case of an amino acid substitution in the amino acid sequence of a polypeptide according to the invention, one or several consecutive or non-consecutive amino acids are replaced by "equivalent" amino acids. The expression "equivalent" amino acid is used herein to designate any amino acid that may be substituted for one of the amino-acids belonging to the native protein structure without decreasing the binding properties of the corresponding peptides to the antibodies raised against the polypeptides of the invention. In other words,

the "equivalent" amino-acids are those which allow the generation or the synthesis of a polypeptide with a modified sequence when compared to the amino acid sequence of the recombinant hSK polypeptides of interest, said modified polypeptide being able to bind to the antibodies raised against the recombinant hSK of interest and/or to induce antibodies recognizing the parent polypeptide.

The peptides containing one or several "equivalent" amino-acids must retain their specificity and affinity properties to the biological targets of the parent protein, as it can be assessed by a ligand binding assay or an ELISA assay.

Examples of amino-acids belonging to specific classes include Acidic (Asp, Glu), Basic (Lys, Arg, His), Non-polar (Ala, Val, Leu, Ile, Pro, Met, Phe, Trp) or uncharged Polar (Gly, Ser, Thr, Lys, Tyr, Asn, Gln) amino-acids.

Preferably, a substitution of an amino acid in a recombinant hSK of the invention, or in a peptide fragment thereof, consists in the replacement of an amino acid of a particular class for another amino acid belonging to the same class.

By an equivalent amino acid according to the present invention is also contemplated the replacement of a residue in the L-form by a residue in the D form or the replacement of a Glutamic acid (E) residue by a Pyro-glutamic acid compound. The synthesis of peptides containing at least one residue in the D-form is, for example, described by Koch (1977).

A specific embodiment of a modified peptide of interest according to the present invention, includes, but is not limited to, a peptide molecule, which is resistant to proteolysis. This is a peptide in which the -CONH- peptide bond is modified and replaced by a (CH₂NH) reduced bond, a (NHCO) retro inverso bond, a (CH₂-O) methylene-oxy bond, a (CH₂S) thiomethylene bond, a (CH₂CH₂) carba bond, a (CO-CH₂) cetomethylene bond, a (CHOH-CH₂) hydroxyethylene bond), a (N-N) bound, a E-alcene bond or also a -CH=CH-bond.

The invention also encompasses a recombinant hSK in which at least one peptide bond has been modified as described above.

The polypeptides according to the invention may also be prepared by the conventional methods of chemical synthesis, either in a homogenous solution or in solid phase. As an illustrative embodiment of such chemical polypeptide synthesis techniques, it may be cited the homogenous solution technique described by Houbenweyl (1974).

The recombinant hSK of interest, or a fragment thereof may thus be prepared by chemical synthesis in liquid or solid phase by successive couplings of the different amino acid residues to be incorporated (from the N-terminal end to the C-terminal end in liquid phase, or from the C-terminal end to the N-terminal end in solid phase) wherein the N-terminal ends and the reactive side chains are previously blocked by conventional groups.

For solid phase synthesis, the technique described by Merrifield (1965a; 1965b) may be used in particular.

H) Antibody production

The recombinant hSK of the invention and its peptide fragments of interest can be used for the preparation of antibodies.

Polyclonal antibodies may be prepared by immunization of a mammal, especially a rabbit, a sheep, a donkey, a horse or a goat with a polypeptide according to the invention that is combined with an adjuvant of immunity, and then by purifying the specific antibodies contained in the serum of the immunized animal on an affinity chromatography column on which has previously been immobilized the polypeptide that has been used as the antigen.

Monoclonal antibodies from mammals especially from mouse or rat may be prepared from hybridomas according to the technique described by Kohler and Milstein (1975)).

The present invention also deals with antibodies produced by the trioma technique and by the human B-cell hybridoma technique, such as described by Kozbor et al. (1983).

Antibodies of the invention also include chimeric single chain Fv antibody fragments (US Patent N° 4,946,778; Martineau et al., (1998), antibody fragments obtained through phage display libraries Ridder et al. (1995) and humanized antibodies (Leger et al., 1997).)

5

D) Assay for the screening of inhibitors of hSK

Sphingosine kinase converts the substrate sphingosine to sphingosine-1-phosphate (S1P). S1P is believed to play several roles in physiological processes. Some of the potential physiological roles of S1P include:

10

1) Within cells:

Release of calcium from stores;

Activation of cyclin-dependent kinases;

Key signalling intermediate in Fc receptor initiated cascades;

15

fMLP induced enzyme release;

TNF- α induced (endothelial cells) adhesion molecule expression; and

Depression of excitability in ventricular myocytes.

Sphingosine kinase appears to play a pivotal role in the activation of the signaling cascade initiated at Fc ξ RI by modulating the balance of the counterregulatory lipids. (Prieschl et al., 1999)

20

Furthermore, PDGF (platelet derived growth factor) induces high levels of sphingosine kinase activity and S1P generation in platelets. (Yatomi et al., 1997; Yatomi et al., 1995)

25

In human umbilical vein endothelial cells, TNF α , a pleiotropic cytokine, induces activation of sphingosine kinase and generation of S1P which turn may serve as a second messenger to mediate TNF α induced endothelial cell activation and adhesion molecule expression. (Xia et al., 1998)

30

Also, in osteoblast sphingosine monophosphate plays a role of second messenger for TNF α induced IL-6 (interleukine 6) synthesis. (Tokuda et al., 1999) These properties strongly indicate a potentially important role of S1P and hence of

sphingosine kinase itself in pain, as well as in inflammation, particularly inflammation following injury.

It has been further shown that S1P protects from apoptosis. More particularly, S1P prevents the appearance of intranucleosomal DNA fragmentation and morphological changes which are main features of apoptosis. (Spiegel et al., 1998)

Furthermore, it has also been demonstrated that S1P is a key mediator of the mitogenic effect of oxLDL (oxidized low density lipoprotein) which have been implicated in diverse biological events leading to development of atherosclerotic lesions. (Auge et al., 1999)

As a result, sphingosine kinase may play a role in conditions such as hemostasis, thrombosis, stroke, atherosclerosis, coronary artery disease and dyslipidemia.

A high cellular concentration of sphingosine acts as a potent inhibitor of the immunoglobulin (Ig)E+ antigen-mediated leukotriene synthesis and cytokine production by preventing activation of the mitogen-activated protein kinase pathway. In contrast, high intracellular levels of sphingosine-1-phosphate, also secreted by allergically stimulated mast cells, activate the mitogen-activated protein kinase pathway, resulting in hexosaminidase and leukotriene release or, in combination with ionomycin, cytokine production. Hence, the balance between sphingosine and S1P modulates the allergic responsiveness of mast cells. (Prieschl et al., 1999)

As a result, inhibitors of sphingosine kinase may be useful in preventing allergy reactions.

It has been previously shown that sphingosine kinase activity is stimulated by tumor promoters such as 12-O-tetradecanoylphorbol-13-acetate. Hence, one may infer that excessive stimulation of sphingosine kinase activity could lead to the development of proliferative diseases. On the other hand, inhibition of sphingosine kinase prevented the survival effect of $1\alpha,25$ -dihydroxyvitamin D₃ ($1,25$ -(OH)₂D₃), a cytoprotective agent, on human promyelocytic leukemia HL-

60 cells. (Kleuser et al., 1998) Thus, sphingosine kinase inhibitors may be useful in the prevention and treatment of proliferative diseases including cancer, hematopoietic disorders such as leukemia.

5 2) When released from the cells:

 Proliferation;

 Chemotaxis (attraction and activation of macrophages);

 Cytoskeletal changes (Stress fiber formation and cell shape contraction, aggregation and secretion);

10 Mediates attachment: Fibronectin matrix assembly; and

 Assembly and phosphorylation of paxillin and p125-FAK.

15 More particularly, sphingosine kinase plays a role in Ca^{2+} release through GPCRs (G-protein-coupled receptors) induced Ca^{2+} signaling. (Meyer et al, 1998)

20 S1P is released from activated platelets in large amounts. (Yatomi et al., 1995) This could indicate a potential role of S1P in thrombosis, hemostasis, the natural wound healing processes, atherosclerosis, stroke, myocardial infarction.

25 Furthermore, S1P stimulates the binding of fibronectin or its N-terminal 70-kd-fragment to cells. Organization of fibronectin into extracellular matrix is a tightly regulated process, mediated by initial reversible binding by the 70-kd N-terminal region of fibronectin to specific cells surface binding sites, followed by insolubilization into fibrils. The adhesive information present after insolubilization of fibronectin is postulated to play a central role in various physiological and pathophysiological processes, including embryogenesis, wound-healing, inflammation, and degenerative disease processes such as atherosclerosis and fibrosis. (Windh et al., 1999)

More particularly, S1P could have a likely role in early atherogenesis and fibrosis. As a consequence, suitable sphingosine kinase inhibition could be useful in the treatment of cardiovascular diseases including atherosclerosis, thrombosis and dyslipidemia, diabetes including type I and type II diabetes and particularly type I diabetes, stroke, autoimmune and inflammatory diseases such as multiple sclerosis, psoriasis, epidermodysplasia verruciformis and inflammatory arthritis, T helper-1 related diseases, chronic obstructive pulmonary disease, asthma, cancer and neurodegenerative disorders.

The isolation of a nucleotide sequence encoding human sphingosine kinase is useful in that it allows the skilled person to screen for suitable sphingosine kinase inhibitors. These inhibitors or structural analogues thereof can be used to treat or prevent one and/or several of the disease states referred to above. The term "structural analogue" is intended to designate compounds which have a common chemical backbone with the initial inhibitors identified through the screening assays of the invention but which bear substituents which have been modified to improve or enhance properties of the initial inhibitors such as biological activity, reduced side effects, enhanced solubility, enhanced bioavailability and the like.

Several assay formats can be used to carry out the method of the present invention. Preferred assay formats include scintillation assays such as the scintillation proximity assay (SPA) or the flashplate assay. Other assay formats well known to those skilled in the arts such as the filter binding assay and the centrifugation assay are also contemplated in the present invention. SPA and flashplate assays are preferred assay formats for the present invention. Additional details on these assays are provided below.

Scintillation assay technology either involves the use of scintillant beads (for the SPA assay) or plates (for the flashplate assay). SPA beads are usually made from either cerium-doped yttrium ion silicate ($\text{Y}_2\text{SiO}_5\text{:Ce}$) or polyvinyltoluene (PVT)

containing an organic scintillant such as PPO. Flashplates commonly used are those such as Ni chelate flashplates although other flashplates can also be used.

5 Assays are usually carried out in aqueous buffers using radioisotopes such as ^3H , ^{125}I , ^{14}C , ^{35}S or ^{33}P that emit low-energy radiation, the energy of which is easily dissipated in an aqueous environment. For example, the electrons emitted by ^3H have an average energy of only 6 keV and have a very short path length ($\sim 1 \text{ } \mu\text{m}$) in water. If a molecule labelled with one of these isotopes is bound to the bead or flashplate surface, either directly or via interaction with another molecule
10 previously coupled to the bead or flashplate, the emitted radiation will activate the scintillant and produce light. The amount of light produced, which is proportional to the amount of labelled molecules bound to the beads, can be measured conveniently with a liquid scintillation (LS) counter. If the labelled molecule is not attached to the bead or a flashplate surface, its radiation energy is
15 absorbed by the surrounding aqueous solvent before it reaches the bead, and no light is produced. Thus, bound ligands give a scintillation signal, but free ligands do not, and the need for a time-consuming separation step, characteristic of conventional radioligand binding assays, is eliminated. The manipulations required in the assays are reduced to a few simple pipetting steps leading to
20 better precision and reproducibility.

In the context of the present invention, one of the preferred embodiments of the assay includes the binding of sphingosine to SPA beads or flashplates. The binding is preferably carried out through BSA although other binding means
25 could be contemplated. The assay medium comprises recombinant hSK and labelled ATP. What is measured is the ability of the candidate ligand to prevent conversion of sphingosine to labelled S1P by phosphorylation of sphingosine using recombinant hSK through labelled ATP. If the candidate ligand inhibits recombinant hSK, conversion of sphingosine will not occur and a signal not
30 substantially different from the background noise signal will be recorded. On the other hand, if no hSK inhibition occurs, sphingosine conversion will take place

and a signal resulting from the interaction between labelled SIP and the flashplate or SPA bead will be recorded.

J) Antisense

Oligonucleotides ie RNA, DNA such as: genomic DNA, cDNA or RNA/DNA hybrid sequences, comprising the antisense strand of the human sphingosine kinase type 1 are used to inhibit in vitro or in vivo the sphingosine kinase expression. Thus the inhibition of sphingosine kinase expression permits the study of the effect of hSK1 in cells, tissues or animals.

K) Knock Out animals

The determination by the inventors of the 80 amino acids between species conserved region present in SK now allows the design of polynucleotide constructs wherein the nucleic acid portion encoding the 80 amino acids conserved region; or a portion of it has been deleted.

In a preferred embodiment the polynucleotide construct as defined above contains a genomic polynucleotide encoding a SK from which at least a part of the nucleic acid portion encoding the 80 amino acids conserved region has been deleted and wherein the deleted nucleic acid portion is replaced by a heterologous polynucleotide sequence.

Said constructs may be included in vectors in order to replace a portion of the naturally occurring sphingosine kinase sequence within the genome of a mammal by homologous recombination.

According to this specific embodiment, such a recombinant vector of the invention may be used to generate knock-out animals, preferably knock-out mammals, most preferably knock-out mice and rats.

In a first embodiment of the nucleic acid above, the genomic polynucleotide encodes a human, a mouse or a rat SK from which the nucleic acid portion encoding the 80 amino acids conserved region or a portion of it has been deleted.

In a second embodiment of the nucleic acid above, the heterologous polynucleotide comprises a selection marker.

In a third embodiment of the nucleic acid above, the heterologous polynucleotide comprises at least a loxP sequence at its 5' end and at least a loxP sequence at its 3' end. The loxP sequence is composed of two palindromic sequences of 13 bp separated by a 8 bp conserved sequence (HOESS et al., 1986).

The recombination by the Cre enzyme between two loxP sites having an identical orientation leads to the deletion of the DNA fragment. The Cre-loxP system used in combination with a homologous recombination technique is described by GU et al. (1993, 1994).

The vector containing the genomic SK sequence in which the sequence encoding the 80 amino acids conserved region or a portion of it has been deleted is designed in such a way that selectable markers are flanked by loxP sites of the same orientation. It is possible, by treatment by the Cre enzyme, to eliminate the selectable markers while relocating the hSK genomic polynucleotide of interest that has been inserted by a homologous recombination event.

Two selectable markers are needed: a positive selection marker to select for the recombination event and a negative selection marker to select for the homologous recombination event. Vectors and methods using the Cre-loxP system are described by ZOU et al. (1994).

In the specific embodiment of the nucleic acids of the invention wherein said nucleic acid comprises the genomic polynucleotide encoding the mouse SK in which the nucleic acid portion encoding the 80 amino acids conserved region or a portion of it has been deleted, the person skilled in the art may advantageously refer to the examples below.

In a further aspect of the invention, a nucleic acid which encodes for a polypeptide as defined above is operably linked to a regulatory sequence.

Preferably, the regulatory sequence consists of a inducible promoter.

Most preferably, the regulatory sequence consists of a promoter inducible by Ponasterone.

EXAMPLES

Material and methods

Growth medium including all supplements were purchased from Gibco BRL (Paris, France). Transfection reagents were from QIAGEN (Paris, France). All lipids were purchased from Sigma-Aldrich (Paris, France). [$\alpha^{32}\text{P}$]ATP and [$\gamma^{33}\text{P}$]ATP were from Amersham Pharmacia Biotech (Amersham, Paris, France). Human poly A+ RNA multiple tissue Northern blots, and the pcDNA3 mammalian expression vector were purchased from CLONTECH (Palo Alto, CA, USA). Restriction enzymes were purchased from New England Biolabs (Beverly, MA, USA). EST-IMAGE clone 1946069 was purchased from UK HGMP Resource Centre (Hinxton Cambridge, UK.)

COS7 cells (Monkey fibroblast cells) were grown in Dulbecco's modified Eagle's medium containing 4,500 mg/L Glucose supplemented with 10% fetal calf serum, 2 mM glutamine, 10 IU/ml penicillin and 10 mg/ml streptomycin at 37°C, 6.5% carbon dioxide in a water saturated atmosphere.

Example 1: Human Sphingosine Kinase (hSK1) cDNA isolation.

Searches using the recently cloned murine sphingosine kinase cDNA sequence (Kohama et al., 1998) identified a human Est cluster and several human Est sequences in public (Unigene) and local (Compugen) cDNA cluster databases. The insert of the IMAGE clone 1946069 a member of the cluster was sequenced and subcloned into the pcDNA3 mammalian expression vector.

The 1.7 kb insert showed high level of similarity (76%) to the mouse SK1a cDNA and covered the entire coding region. Peptide sequence alignment of mouse and human sequences and the biological activity of the expressed enzyme suggest that the insert of the IMAGE clone 1946069 harbors the coding region of the human SK cDNA. This is in agreement with human partial peptide sequences, deduced from Est sequences by Kohama et al (Kohama et al., 1998).

The cDNA sequence and peptide sequences of hSK1 are shown in Figure 1. The open reading frame of the cDNA is 1155 nt. The translational initiator ATG is in a partial Kozak consensus (Kozak, 1987).

5 The PCR primers are the following :

Primers A and B:

A= 5'end TAT GCT AGC ATG GAT CCA GCG GGC GGC (SEQ ID NO:
4)

10

B= 3'end AAT GAA TTC TCA TAA GGG CTC TTC TGG (SEQ ID NO:
5)

Primers C and D:

15 C= 5'end TTA GAA TTC CAC CAT GGA TCC AGC GGG CGG C (SEQ
ID NO: 6)

D= 3'end ATT ATC GTC GAC TAA GGG CTC TTC TGG CGG (SEQ ID
NO: 7)

20

These primers are used for the cDNA amplification such as PCR amplification.

Example 2: Sphingosine kinase characterization

25

The predicted peptide sequence is 384 aa (seq ID N°3), with a predicted mass of 42.5 kD and pI of 6.9 at neutral pH. Similarity to the mouse SK1a is 85% (Needleman Wunsch similarity index). With the exception of the C terminal, similarity with the mouse SK is contiguous.

30

Peptide similarity searches identify a 80 aa conserved region (Arg16 -Pro95) (SEQ ID N° 8) present in various known and hypothetical peptides from bacteria

to human. (CAB14972 *Bacillus subtilis*; CAA18718 *Arabidopsis thaliana*; CAB11477 *Saccaromyces pombe*; S51398 *Saccaromyces cerevisiae*; SS67059 *Saccaromyces cerevisiae*; CAA91259 *Caenorhabditis elegans*; AAC67466 *Caenorhabditis elegans*; Mouse SK1a (Mouse SPHK 1a); hSK1 (huSPHK)). The conserved amino acids are shown in black (Figure 2B)

This sequence includes a region distantly related to a short signature peptide, LVRSEELGRWDALVVM (SEQ ID N°9) of NADPH dependent aldo-keto reductase family of enzymes. Within the 80 aa conserved region, highly conserved residues mark seemingly characteristic and predictable features of the secondary peptide structure in three blocks. (Figure 2A) Conserved Asn22-Pro23 and Gly26 residues present a probable beta turn and a coil structure, proximal to the GGK GK sequence (SEQ ID NO: 24) which may be part of the ATP binding site also suggested for the mouse SK1 (Kohama et al., 1998). His59-Ala60 are indicated to be exposed on the surface, while Gly80-Asp81-Gly82 suggest the presence of a flexible region. Spacing of Asn22-Pro23, and Gly26, in block one, Thr50, His59-Ala60, in block two and Gly80-Asp81-Gly82, Glu86 and Gly90 residues in block three of the conserved region is identical from *Bacillus subtilis* to human.

Example 3: Transfection of hSK1.

COS7 cells were transiently transfected with the vector pcDNA3 alone or vector containing the human sphingosine kinase cDNA, using the Qiagen reagent, SuperFect. Cells were seeded 5×10^6 per well, in 6 wells plates. After 24 hrs, cells were transfected with 10 μ g of vector (pcDNA3) mixed with 20 μ l SuperFect, or with 10 μ g vector containing the human sphingosine kinase cDNA (pcDNA3-hSK1) mixed with 20 μ l SuperFect.

Example 4: Sphingosine kinase activity and specificity assay.

Sphingosine kinase activity was assayed as previously described (Kohama et al., 1998). Briefly, sphingosine kinase activity was determined, by incubating cell extracts for 30 min at 37°C, in the presence of 50 µM sphingosine, 0.25% Triton X-100, and [³³P]ATP (10 µCi, 1 mM), and MgCl₂ (10 mM). The kinase activity was expressed as nanomoles of SPP/min/mg.

• hSK1 activity and substrate characterization.

To ensure that the hSK was indeed a functional sphingosine kinase, COS7 cells were transfected with the vector-pcDNA3-containing the hSK1 cDNA and, after 48 hours, sphingosine kinase activity was measured. Low levels of endogenous sphingosine kinase activity were present in control cells (either untransfected or transfected with vector alone). However, cells transfected with hSK (with 10 µg DNA) generated over 107-fold increased sphingosine kinase activity (Figure 3).

Figure 3 shows that hSK1 specifically phosphorylates D-*erythro*-sphingosine (D-*erythro*-SPH), and to a lesser extent D,L-*erythro*-dihydrosphingosine (D,L-*erythro*-DHS). This kinase does not phosphorylate: any of the "*threo*" isoforms of dihydrosphingosine (D,L-*threo*-dihydrosphingosine; L-*threo*-dihydrosphingosine; L-*threo*-dihydrosphingosine); ceramides (hydroxy-ceramide; non-hydroxy-ceramide); diacylglycerol (DAG); phosphatidylinositol (PI); phosphatidylinositol-4-phosphate (PIP); or phosphatidylinositol-4,5-bisphosphate (PIP₂).

The substrate specificity of the expressed hSK was found to be similar to purified rat sphingosine kinase (Olivera et al., 1998), and to the recently cloned mouse sphingosine kinase (Kohama et al., 1998). The best substrate was D-(+)-*erythro*-sphingosine, followed by the D,L-*erythro*-dihydrosphingosine, which was phosphorylated to 50% of the observed phosphorylation levels achieved for D-(+)-*erythro*-sphingosine.

- Substrate specificity and competitive inhibition of the hSK1.

The expressed hSK1 shows typical Michaelis-Menten kinetics ($v_{\text{Max}} = 56$ nMole/min/mg and $K_m = 5\mu\text{M}$) (Figures 4A (4A1, 4A2) and 4B (4B1, 4B2)). D,L-threo-dihydrosphingosine (on figure 4A (4A1, 4A2), DHS) and N,N-diMethyl-sphingosine (N,NdiMS) are known inhibitors of sphingosine kinase (Kohama et al., 1998; Olivera et al., 1998). In agreement to this, we show here that both these compounds inhibit expressed hSK1 activity. The kinase is inhibited by D,L-threo-dihydrosphingosine ($K_i = 3\mu\text{M}$), and N,N-diMethyl-sphingosine ($K_i = 5\mu\text{M}$).

Example 5: hSK constructs fused to EGFP (Enhanced Green Fluorescence Protein)

In order to characterise and understand the potential mechanisms that regulate hSK1 activity and cellular localisation, we have made two hSK constructs fused to EGFP (Enhanced Green Fluorescence Protein), at either end of the kinase.

- Construction of EGFP-hSK1. (N-terminal fusion) mammalian expression.

A PCR reaction was carried out using the pcDNA3-hSK1 construct as a template, and primers designed to amplify the coding sequence of hSK1 at the same time inserting cloning restriction sites at both ends (NheI - EcoRI) in order to align the EGFP with the hSK1 and make the fusion protein in frame. The constructs carries the EGFP at the N-terminus of the hSK1.

Primers A and B:

A= 5'end TAT GCT AGC ATG GAT CCA GCG GGC GGC (SEQ ID NO:
4)

B= 3'end AAT GAA TTC TCA TAA GGG CTC TTC TGG (SEQ ID NO:5)

• Construction of EGFP-hSK1. (C-terminal fusion) mammalian expression.

A PCR reaction was carried out using the pcDNA3-hSK1 construct as a template, and primers designed to amplify the coding sequence of hSK1 at the same time inserting cloning restriction sites at both ends (EcoRI - SalI) in order to align the hSK1 sequence with the EGFP and make the fusion protein in frame. The constructs carries the EGFP at the C-terminus of the hSK1.

Primers C and D:

C= 5'end TTA GAA TTC CAC CAT GGA TCC AGC GGG CGG C (SEQ ID NO: 6)

D= 3'end ATT ATC GTC GAC TAA GGG CTC TTC TGG CGG (SEQ ID NO: 7)

• Transfection of hSK1.

COS7 cells were transiently transfected with the vector pcDNA3 alone or vector containing the human sphingosine kinase cDNA, using the Qiagen reagent, SuperFect as described in example 4.

• Transfection of EGFP-hSK1. (N-terminal fusion)

COS7 cells were transiently transfected with the vector pCI-EGFP1 alone or vector containing the human sphingosine kinase cDNA (see figure 14), using the Qiagen reagent, SuperFect. Cells were seeded 5×10^6 per well, in 6 wells plates. After 24 hrs, cells were transfected with 10 μ g of vector (pCI-EGFP1) mixed with 20 μ l SuperFect, or with 10 μ g vector containing the human sphingosine kinase cDNA (pCI-EGFP1-hSK1) mixed with 20 μ l SuperFect.

Figure 14 shows the vector for the construction of hSK-EGFP (N-terminal) fusion for expression in mammalian cells. pCI-EGFP size is 4724bp. EGFP coding sequence (716pb) was amplified with EGFP.XbaI (sens) and STOP.EGFP (antisens) primers, cut XBAI/XhoI and subcloned into pCI cut by NheI and XhoI. EGFP STOP codon was deleted. The frame for subcloning a sequence of interest with EGFP fused to the N-terminus is shown at the bottom of the figure.

• Transfection of hSK1-EGFP. (C-terminal fusion)

COS7 cells were transiently transfected with the vector pCI-EGFP-2 alone or vector containing the human sphingosine kinase cDNA (see figure 15), using the Qiagen reagent, SuperFect. Cells were seeded 5×10^6 per well, in 6 wells plates. After 24 hrs, cells were transfected with 10 μ g of vector (pCI-EGFP-2) mixed with 20 μ l SuperFect, or with 10 μ g vector containing the human sphingosine kinase cDNA (pCI-EGFP-2-hSK1) mixed with 20 μ l SuperFect.

Figure 15 illustrates the vector for the construction of hSK-EGFP (C-terminal fusion) for expression in mammalian cells. pCI-EGFP2 size is 4733 bp. EGFP coding sequence (725bp) was amplified by PCR with EGFP2-TOP (sens) and EGFP2-BOTTOM (antisens) primers, cut XhoI/NotI and subcloned into pCI cut by SalI and NotI. A new SalI site was included into the PCR product. The frame for subcloning a sequence of interest with EGFP fused to the C-terminus is shown at the bottom of the figure.

The two fusion proteins express well in COS7 cells, the EGFP/hSK1 fusion protein expresses primarily as a soluble cytosolic protein. (Figures 5A and 5B)

Figure 5 A describes the expression and cellular localisation of hSK1 fused with EGFP at the N-terminal end. EGFP-hSK1 (N-terminal fusion) is expressed in a cytosolic manner when transfected into Cos7 cells, as shown by the green colour (lightest colours on figure 5A).

5 Whereas, the hSK1-EGFP appear to be partially localised in a granular form, although, general cytosolic expression is also observed (Figure 5B).

Figure 5B illustrates the expression and cellular localisation of hSK1 fused with EGFP at the C-terminal end. hSK1-EGFP (C-terminal fusion) is expressed primarily in a cytosolic manner, with some granular localisation when
10 transfected into Cos 7 cells, as shown by the green colour (lightest colours on figure 5B).

Kinase assays, of cell extracts from cells transfected with either construct, show that the EGFP-hSK1 fusion protein is more active than the hSK1-EGFP one.
15 (Figure 6)

Figure 6 shows the kinase activity of hSK fusion proteins. Overexpression of hSK-EGFP (N-terminal fusion) (EGFP-hSK1) has similar activity as the overexpressed unfused untagged protein. On the other hand, the hSK-EGFP C-terminal fusion (hSK1-EGFP) shows 40% less activity than the unfused or N-terminal fusion proteins.
20

This is not a problem with transfection efficiency, since Western blots (Figure 7), as well as the confocal images (figures 5A and 5B), indicate that the levels of expression for the two proteins is similar.

25 Figures 5A and 5B show similar green fluorescence intensity suggesting that the expression levels for both C-terminal and N-terminal fusion proteins are similar.

Figure 7 is a Western blot analysis with anti-EGFP Antibody. Figure 7 demonstrates that both C-terminal (hSK1-EGFP) and N-terminal (EGFP-hSK1) hSK/EGFP fusion proteins are expressed to similar levels in Cos7 cells.
30

Example 6: Sphingosine kinase localisation in tissues

A Northern blot containing approximately 1 µg of poly A+ RNA per lane from 12 different human tissues, was hybridised with the 1.7 kb insert of pcDNA3-hSK1, purified from the gel and labeled with [³²P]αATP using random primer labeling kit. The hybridization using ExpressHyb™ buffer (CLONTECH), was carried out according to the manufacturer instructions. The bands were visualized by autoradiography and quantified by densitometer.

The tissue distribution of hSK1 mRNA expression in human tissues was analyzed by Northern blotting (Figure 8).

Figure 8 shows the tissue distribution of hSK1 messenger RNA. Premade Northern blot containing approximately 1 µg of poly A+ RNA per lane from 12 different human tissues, was hybridized as described under methods. The numbers at the bottom of each line indicate the expression levels relative to background, and were quantified by densitometry.

This revealed highest expression in adult lung (46 fold over background) and spleen (38 fold), followed by peripheral blood leukocytes (30 fold), thymus (28 fold) and kidney (24 fold), it is also expressed in brain (12 fold), and heart (11.5 fold). Low levels of expression are observed in skeletal muscle (2.6 fold), colon (2 fold), liver (1.8 fold), small intestine (1.2 fold), and placenta (1.3 fold). The tissue distribution and expression levels of hSK1 mRNA are overall very similar to that reported for the murine homologue (Kohama et al 1998). However, in both mouse and human, mRNA levels in the liver are low, and this contrasts to the finding that in the rat liver SK enzyme activity is twofold elevated compared to the brain (Olivera et al 1998). However, mRNA levels for SK have not been reported in the rat. In addition, data base searches, with the stSG2854 marker suggest expression in endothelial cells, retinal pigment epithelium, and senescent fibroblasts.

Example 7: Genomic localisation of sphingosine kinase, related diseases:

Several members of the Unigene cluster Hs.68061 have been mapped. Sequence identity of these Ests with hSK and the mapping data indicate that the gene is located in chromosome 17q25.2 band in a 9 cM interval between microsatellite markers D17S785 and D17S836 (104.7 and 114 cM respectively). The interval includes an STS (stSG2854), identical with Est sequences of the Hs.68061 UniGene cluster.

The cloning of the hSK1 is an important step towards the elucidation of the role this enzyme plays in signal transduction pathways mediated by a wide range of receptor coupled mechanisms. Moreover, several members of the Unigene cluster Hs68061 have been mapped. An approximately 50 cM region, on 17q25, which harbors the stSG2854 has been implicated in several autoimmune and inflammatory diseases, such as multiple sclerosis (Kuokkanen et al., 1997), psoriasis and epidermodysplasia verruciformis (Nair et al., 1977; Tomfohrde et al., 1994; Enlund et al., 1999; Ramoz et al., 1999), and by synteny homology, in a rat model of inflammatory arthritis (Remmers et al., 1996). Linkage in psoriasis has been reported by multiple independent groups. Together, these data identify a shared autoimmune / inflammatory region described recently by Becker et. al. (Becker et al., 1998). Because of its expression pattern and biology, SK is a possible disease susceptibility gene candidate in this region. As a result, the invention also concerns a method for detecting a mammal's susceptibility to develop auto-immune and inflammatory diseases which comprises comparing said mammal's DNA sequence encoding SK1 to the DNA sequence of SEQ ID NO: 1 or SEQ ID NO: 2 and determining the presence of single nucleotide polymorphism or polymorphic region in said mammal's coding sequence encoding SK1.

Example 8: Sphingosine kinase expression in insect cells

- **Isolation of recombinant Bacmid DNA preparation**

Sphingosine kinase cDNA was cloned into a pFasbac HT donor plasmid according the manufacturers instructions (Gibco BRL, Gaithersburg, MD). FastBac plasmid DNA and sphingosine kinase cDNA was prepared by digesting 1 µg DNA with the selected restriction endonucleases under appropriate conditions. The insert fragment was ligated into the prepared pFasbac HT vector downstream from histidine tag under appropriate conditions. The recombinant plasmid pFastBac donor plasmid was transformed into DH10Bac, (instructions (Gibco BRL, Gaithersburg, MD) for transposition into the bacmid. Isolation of recombinant bacmid DNA was selected by PCR of the sequence desired on whites colonies. Preparation of DNA bacmid was performed under conditions specifically developed for isolating large plasmids (> 100 Kb) and adapted for isolating bacmid DNA (Quiagen).

- Recombinant protein expression in Sf21 cells

Sf21 cells were transfected with the recombinant bacmid DNA in presence of Cellfectin reagent. Cell culture, recombinant virus purification and titration of the viruses were performed according the manufacturers instructions (Gibco BRL, Gaithersburg, MD). For protein expression, cells at a density of 2x10⁶/ml were infected with the recombinant virus at an MOI of 5 to 10. Three days post infection, cells were pelleted by centrifugation and harvested in homogenization buffer (Bis Tris 20mM (pH6.5), EDTA 10mM, DTT 2.5 M) supplemented with a mixture of protease inhibitors (Boehringer). Glycerol was added to a final concentration of 20-30% to all homogenates that were then stored at - 20°C in aliquots. The gene has been cloned into a pFastbac HT expression vector, the expressed protein will contain 6X his at its amino terminus allowing the desired protein to be purified. The fusion protein was purified with a appropriate based buffer system using NI-NTA resin.

- Partial purification

HSK1 was subcloned into the Baculovirus shuttle vector pFastBacHTa which incorporates the sequence for a 6x histidine affinity tag onto the N-terminus. This Baculovirus construct was mixed with viral DNA and introduced into Sf21 insect cells. New recombinant Baculovirus was isolated by plaque purification and five isolates examined for protein production. The best isolate was chosen and high titre viral stock generated. Sf21 insect cells were infected and the cells harvested 60 hours post infection. Expression of hSK1 was confirmed by Westernblot. Infected Sf21 insect cells from a culture were used for partial purification of hSK1 using Nickel beads.

hSK1 was sub-cloned into pFastBacHTa (Life technologies Cat No 50322, lot No KDW704) using restriction site BamHI and PstI. This construct was confirmed by sequencing.

The recombinant hSK1 in pFastBacHTa was used to transform DH10Bac E. Coli (which contains the bacmid shuttle vector bmon14272). Transposition from donor plasmid to acceptor shuttle plasmid was detected by blue/white colony selection on X-gal/IPTG plates. White colonies were selected, grown up and recombinant Bacmid purified bac-to-Bac Baculovirus expression systems. (Instruction manual Gibco BRL Life Technologies)

Sf21 cells for viral stocks (NERC,Oxford) were grown in 47.5% TC100 (from Life Technologies; cat No 13055-025; lotNO 3031505)+5% heat-inactivated North American foetal bovine serum (FBS; from Life technologies; cat NO 10085-140; lot No 06Q6073A) as suspension cultures in shaker flasks and attached using standard procedures (King et al. ,1992). Sf21 cells in ExCell 401 (lot No 9N3936) were transfected with purified recombinant bacmid DNA in the presence of Lipofectin (Life Technologies) using standard methods (King et al. ,1992). The culture medium was collected 7 days post-transfection.

A monolayer of Sf21 cells (3.5x10⁶ cells/60mm dich) was infected with serial dilutions of the viral stocks from the transfection mix described above, overlaid

with a mixture of Graces Insect Medium (2x; Life Technologies), 10% FBS (Life Technologies; heat inactivated North American; cat No 10085-140; lot No06Q6073A); 1.5% SeaPlaque Agarose (lowgen) solution and stained with neutral red (6% in PBS; Sigma) 4 days post-infection. (King et al, 1992)

5

Figure 17 shows an electrophoresis gel of the partial purification of hSK1 from Sf21 insect cells.

150ml of Sf21 insect cells were infected at an MOI 10 and cells collected 60hr post infection. Samples were prepared and binding to aNi column carried out.

10

10µl from column sample eluates were mixed with 5X reducing SDS/PAGE buffer and 15µl loaded per well on 4-12% NuPAGE Bis-Tris Gel. The electrophoresis gel was stained with Coomassie Blue.

1=Total cell lysate

2=Lysate following low speed spin

15

3=Flowthrough column

4-6=Column wash fractions 1-3

7-11=Column elution fractions 1-5

The single band of line 8 corresponds to the predicted molecular weight of hSK1 ie around 43kD. The addition of the column elution fractions 7 and 8 represents the partial purified hSK1.

20

Example 9: Sphingosine kinase expression in bacteria

- **Construction of GST-hSK1. (N-terminal fusion) bacterial expression.**

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A PCR reaction was carried out using the pcDNA3-hSK1 construct as a template, and primers designed to amplify the coding sequence of hSK1 at the same time inserting cloning restriction sites at both ends (EcoRI - XhoI), in order to align the PGEX vector containing GST with the hSK1 and make the fusion protein in frame (figure 16). The construct carries the GST at the N-terminus of the hSK1.

30

Primers E and F:

E= 5'end TTA GAA TTC CAC CAT GGA TCC AGC GGG CGG C (SEQ
ID NO: 10)

5 F= 3'end AGT CGA GGC TGA TCA GCG AG (SEQ ID NO: 11)

Figure 16 illustrates the vector for the construction of hSK1 tagged with GST for expression in bacterial cells. The PGEX-SX-3 vector (from Promega) was used to construct and express a hSK1-GST fusion protein in bacteria.

10

- E. Coli cells

E. Coli competent cells (strain BL21) were purchased from Promega
Bacterial transformation was carried out as per supplier Standard
Transformation Protocol.

15

Frozen competent cells were thawed on ice for 5 minutes, 100µl was transferred to a child culture tube. 50ng of hSK1-cDNA was added and mixed by flicking the tube. The tubes were returned to ice for 10 minutes, after which a heat-shock was performed by placing the tubes in a water bath at 42°C for 45 seconds. Immediately the tubes were placed on ice for 2 minutes. 900µl of cold SOC medium was added to the transformation reaction and incubated for 60 minutes at 37°C with shaking. Aliquots of cells were plated on antibiotic containing plates and incubated at 37°C for 12-14 hours.

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25

Example 10: Sphingosine kinase optimized source for the screening assays

- Sphingosine kinase source choice

Several sources of hSK have been tested. Figure 9 illustrates the comparison of hSK activity from different sources: CHO cells, Bacteria, partially purified hSK1 from insect cells. Similar levels are observed in mammal (Cho) and bacterial

30

(BL21) cell extracts. There is a dose response effect of increasing levels of partial purified hSK from insect cell transfections.

As in figure 9 (Y axis is the enzyme activity in SPA cpm), 2 μ g total protein from bacteria (Bl 21) and 0.1 μ g of partially-purified baculovirus/insect cells hSK gave a good signal to noise ratio (around 12 fold).

The total amount of protein needed is 400 mg for 2000 plates, which represent around 10 liters of transformed and induced bacteria.

Experiments have been carried out in an effort to identify the best possible solution for the generation of enough recombinant hSK to run a High Throughput Screening (HTS). Since the mammalian transient expression presents many difficulties to generate enough enzyme for the entire HTS, different bacterial expression systems have been tried, as well as, the baculovirus expression system in insect cells. The results are expressed in Figure 10.

Figure 10 illustrates the comparison of hSK1 activity from different sources: Cos7, bacteria, insect cells. BL21 Transf. Basal means BL21 transfected without IPTG induction. BL21 Tmas. Induced means BL21 transfected with IPTG induction. P.Pur.rSPHK means partial purified recombinant hSK1.

40 μ g of the total cells extract from transfected cos7 cells shows 50% more activity than 40 μ g of total transfected bacterial cell extract. 40 μ g of insect cell extract shows minimal hSK1 activity over basal levels (Cos7 basal). However 6 μ g of partially purified hSK1 from insect cell shows a 3 fold increase over the transfected COS7 cell extract.

Transfected COS7 represent our positive control for optimal activity. The partial purified enzyme from the baculovirus system gives the maximal activity observed thus far. However, with similar amounts of total cell extracts, the bacterial extracts that overexpress the hSK gives between 40% to 50% of the

total activity observed with the mammalian system, which renders this system as the most attractive solution.

• Sphingosine kinase bacterial source optimization

Experiments have been carried out in order to generate enough recombinant human sphingosine kinase for the HTS. Transfected BL21 bacteria, grown at 25°C overnight, yielded active recombinant hSK1, as shown by enzymatic activity of total protein extract. The production has been scaled up to generate over 400mg of total bacterial protein that shows very good levels of SK activity.

In order to solve the solubility/expression problem observed for the recombinant GST-tagged hSK1, we set up a wide range of conditions for bacterial growth and for the induction of protein expression. Thus, growing the bacteria at RT° for 20 hours, and inducing protein expression with 50 µM IPTG, appear to be the optimal condition for the expression of significant amounts of active/soluble recombinant GST-tagged- hSK. (Figure 11)

Figure 11 describes the bacterial growth conditions for optimization of actively expressed hSK1. Different concentration of IPTG for induction, different temperatures of growth (R°T means room temperature), different incubation times are tested.

Furthermore, the bacterial cell extract under optimal bacterial growth and induction conditions (50µM IPTG for 20hr) has 40% activity of the maximal activity observed for transfected mammalian cells (Cos cells) (Figure 12)

Figure 12 shows the comparison of hSK1 activity expressed under different bacterial growth conditions and expressed in Cos cells.

Example 11: Sphingosine kinase antisense oligonucleotides

In order to demonstrate the physiological role of sphingosine kinase 1 in intracellular signalling pathways in immune cells, an antisense oligonucleotide, corresponding to the first 21 coding nucleotides of the hSK1, was designed in an attempt to downregulate the protein and hence its activity. U937 cells were

transfected with the antisense oligo, and calcium signals were analysed in a receptor coupled model in which we have previously shown sphingosine kinase to be activated. Here we show that, in the antisense treated cells the release of calcium from intracellular stores is impaired, demonstrating that sphingosine kinase does indeed play a significant role in receptor-coupled triggered physiological responses.

- Construction of an antisense oligonucleotide against the hSK1

An antisense sequence to the first 24 nucleotides (coding for the first 8 amino acids) of the hSK1-cDNA, (said antisense having the sequence: GGG GCC GCC CGC CGC TGG ATC CAT, SEQ ID NO: 12), was synthesised and protected at both ends with Phosphorothioate linkages for the first and last two nucleotide pairs.

A control "scrambled oligo" (CTGGTGGGAAGAAGAGGACGTCCAT, SEQ ID NO:13) was synthesised and protected at both ends with Phosphorothioate linkages for the first and last two nucleotide pairs.

- Transfection of antisense oligonucleotide to hSK1.

U937 cells were transiently transfected with an antisense oligonucleotide against the first coding 21 nucleotides (coding for the first 7 amino-acids) of the human sphingosine kinase cDNA, using the Qiagen reagent, SuperFect. Cells 1×10^6 per ml, in 10 ml. After 24 hrs, cells were transfected with 2 μ g of scrambled antisense oligo (control) mixed with 20 μ l SuperFect, or with 2 μ g antisense oligo against human sphingosine kinase mixed with 20 μ l SuperFect.

- Protein analysis of hSK1 in U937 cells and the effect of the antisense.

Figure 13 illustrates the physiological relevant role of hSK1 proven by the use of an antisense oligonucleotide.

FCγRI triggered calcium signal in U937 cells (control) was inhibited in cells treated for 48 hrs with an antisense against hSK1.

Figure 18 illustrates the antisense downregulation of hSK1 protein levels.

The blot has been probed with a polyclonal antibody against hSK1 (Ab 0144).

1= Background

2= U937 untreated cell extract 100mg (taken as 100% of expression)

3= U937 antisense treated cell extract 100 mg (12% compared to untreated cells)

This figure shows that the antisense reduces SK1 protein expression level by 88%.

Example 12 : Sphingosine kinase knock out Mouse

A high density filter set of BAC colonies from the 129 mouse strain BAC library RPC22 (Research Genetics) has been screened with the following radiolabelled oligonucleotide probes.

SK5'end49 (gene proximal) CTGGGTCTTGTAGAAGAGCAGCAAGTGCT (SEQ ID NO: 14)

SK5'end48 (gene proximal)

AGTTCACCTGCAATCCTTTCTTATCTGGGTTCG (SEQ ID NO: 15)

SK3'end (gene distal) TTCTGTGGATGGAGAGCTGATGGTATGG (SEQ ID NO: 16)

SK BOX (conserved region) ATGAAGTGGTGAATGGGCTAATGGAACG (SEQ ID NO: 17)

The oligonucleotide probes were derived from the mouse SK1 cDNA sequence (Kohama et al., 1998). Based on multiple alignments of SK1 related cDNA sequences (Melendez et al., 2000), oligonucleotides were selected from the two ends of the cDNAs (gene proximal and gene distal probes) and from the conserved region .

Positive BAC clones were purchased (Research Genetics) and have been re-screened with gene distal and conserved region probes. No clones were found positive with both conserved region and 3' (gene distal) radiolabelled oligonucleotide probes in hybridization experiments.

5

Positive clones (with conserved region probes) :

83 B 4 , 442 C 20 , 424 E 5 , 46 M 1 , 270 B 3 , 225 D 6.

3'end (gene distal probes) positive clones: 61 K 3 , 166 L 16 , 126 H 16 , 69 D 8 , 24 O 6 , 203 P 6 , 224 A 21 , 84 A 12 , 387 G 19 , 545 H 5 , 431 O 19 , 224 A 23

10

The catalytic domain of the enzyme presumably lies in the highly conserved region (see SED ID N°8), which is between aa16 and aa95 in the peptide sequence (SEQ ID NO: 3) downstream of the presumed alternative first exon coded sequences, therefore, this highly conserved region will be targeted in ES cells.

15

Catalysis critical region of the human SK are determined by 5' and 3' truncations and internal deletions. Mouse BAC clones are identified by screening BAC libraries. Mini-libraries are prepared from verified positive clones and these libraries are screened with oligonucleotide probes to obtain genomic fragments that code for the catalytic domain. Sequencing verifies the presence of catalysis critical exons on one genomic fragment. 5' and 3' flanking genomic fragments with appropriate size (2.5-5 Kb) are cloned with oligo-probes, or are PCR amplified with appropriate primers from the cDNA. These fragments are inserted into the pSV-loxP targeting vector, in reverse orientation to the NEO transcription unit (experiment A). In alternative experiments (B) loxP sites are inserted flanking the catalysis critical exon containing genomic fragments and these is also cloned immediately adjacent to the Neo transcriptional unit, the region is flanked with the 5' and 3' homology arms for targeting. Appropriate restriction sites are inserted in order to create an optimal situation for the

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detection of recombination mediated replacement of the wild type catalysis critical region by the loxP site flanked fragment.

5 The targeting vectors are introduced into ES cells by electroporation or other methods. Neomycin resistant colonies are screened for the identification of specific targeting events. In a possible variant of the experiment transient Cre recombinase expression in ES cells are used to remove the loxP flanked Tn-5 Neomycin resistance gene from the targeted allele in experiment A. Once ES cell colonies with targeted alleles are identified blastocysts will be injected with ES
10 cells from these colonies. Mice with high degree of ES cell contribution are screened by coat colour examination, germline transmitting mice will be selected by breeding and tail DNA testing. Once hemizygous targeted mice (SK -/+) are obtained, they are tested in biological experiments together with homozygous null allele (SK -/-) mice (if these are viable) are generated by breeding (Gene targeting, Ed. A.L. Joyner IRL press/Oxford, 1993). In experiment B; homozygous insertion positive mice are generated and crossed with tissue specific Cre recombinase expressing transgenic mice. The result of this
15 experiment is tissue specific deletion of the SK gene. If the Cre recombinase is controlled by an inducible promoter, deletion of SK is inducible

Example 13 : hSK polyclonal antibodies

Four peptide sequences were selected for their apparent hydrophobicity properties, and synthesised.

25 Peptide 1: FTLMLTERRNHARELVRSEE (SEQ ID NO: 18)
Peptide 2: VNGLMERPDWETAIQKPLCS (SEQ ID NO:19)
Peptide 3: ADVDLESEKYRRLGEMRFTL (SEQ ID NO:20)
Peptide 4: SGCVEPPPSWKPPQQMPPPEE (SEQ ID NO:21)

30 Two rabbits were immunised for each peptide giving rise to eight peptide derived polyclonal antibodies (two for each peptide).

Peptide 1: Serum # 0140 (rabbit 1); Serum # 0141 (rabbit 2).

Peptide 2: Serum # 0142 (rabbit 1); Serum # 0143 (rabbit 2).

Peptide 3: Serum # 0144 (rabbit 1); Serum # 0145 (rabbit 2).

Peptide 4: Serum # 0146 (rabbit 1); Serum # 0147 (rabbit 2).

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Claims

- 1) A purified or isolated nucleic acid encoding a human sphingosine kinase.
- 2) A purified or isolated nucleic acid according to claim 1, comprising a
5 polynucleotide having at least 90% identity with the sequence of SEQ ID
NO:1, or SEQ ID NO:2 or a sequence complementary thereto.
- 3) A purified or isolated polynucleotide encoding a human sphingosine kinase
having at least 80% amino acid identity with the sequence of SEQ ID NO:3.
- 4) A purified or isolated polynucleotide of SEQ ID NO: 22 or a polynucleotide
10 hybridizing therewith.
- 5) A purified or isolated polynucleotide comprising at least 10 consecutive
nucleotides of the nucleotide sequence of SEQ ID NO:1, or SEQ ID NO:2.
- 6) A purified or isolated polynucleotide comprising at least 10 consecutive
nucleotides of the nucleotide sequence of SEQ ID NO: 22.
- 7) A purified or isolated polynucleotide according to claim 5 comprising the
15 sequence of SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO:7,
SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID
NO: 16, SEQ ID NO: 17.
- 8) A recombinant vector comprising a nucleic acid as defined in claim 1 to 7.
- 9) A recombinant vector comprising a nucleic acid according to claim 8 which,
20 is a bacterial vector.
- 10) The recombinant vector comprising a nucleic acid according to claim 9
which is a pGEX vector.
- 11) A recombinant vector comprising a nucleic acid according to claim 10 which
25 is a baculovirus vector, preferably pFastBacHTa.
- 12) A recombinant vector comprising a nucleic acid according to claim 8 which
is an eucaryotic vector.
- 13) The recombinant vector comprising a nucleic acid according to claim 12
which is chosen among pcDNA3, pFLAG and pCMV.

14) A recombinant host cell comprising a nucleic acid as defined in claim 1 to 7.

15) A recombinant host cell comprising the recombinant vector of any of claims 8 to 13.

16) An oligonucleotide comprising the antisense strand of a nucleotide according to anyone of claims 1 to 7.

17) An oligonucleotide according to claim 16 having the sequence of SEQ ID NO: 12.

18) A transgenic mammal comprising a nucleic acid according to claim 1 to 7.

19) A transgenic mouse comprising a nucleic acid according to claim 1 to 7.

20) A purified or isolated recombinant polypeptide comprising the amino acid sequence of human sphingosine kinase as defined in claim 1 to 7.

21) A recombinant polypeptide according to claim 20, having at least 80% amino acid identity with a polypeptide of SEQ ID N°3, or a sequence complementary thereto.

22) A purified or isolated recombinant polypeptide comprising the amino acid sequence of SEQ ID NO: 8.

23) A method for amplifying a nucleic acid encoding a hSK as defined in claim 1 to 7, said method comprising the steps of:

(a) contacting a test sample suspected of containing the target hSK nucleic acid, a fragment or a variant thereof, or a sequence complementary thereto, with an amplification reaction reagent comprising a pair of amplification primers as defined in claim 4 to 7 which can hybridize under stringent conditions, the hSK nucleic acid region to be amplified, and

(b) optionally, detecting the amplification products.

24) A kit for amplification comprising :

(a) a pair of oligonucleotide primers as defined in claim 4 to 7 which can hybridize, under stringent conditions to the hSK nucleic acid to be amplified;

(b) optionally, the reagents necessary for performing the amplification reaction.

5 25) A method for producing amino acid sequence, preferably the sequence of SEQ ID NO:3 comprising the steps of :

(a) inserting the nucleic acid as defined in claim 1 to 7 encoding the desired amino acid sequence in an appropriate vector as defined in claim 8 to 13,; or in a host cell as defined in claim 14 to 15;

10 (b) culturing, in an appropriate culture medium, a host cell previously transformed or transfected with the recombinant vector of step (a);

(c) harvesting the culture medium thus obtained or lyse the host cell, for example by sonication or osmotic shock;

15 (d) separating or purifying, from said culture medium, or from the pellet of the resultant host cell lysate, the thus produced recombinant polypeptide of interest, eventually tagged.

26) An antibody directed against a polypeptide as defined in claim 20 to 22.

27) A method for the screening of candidate molecules which are inhibitors of hSK; the said method comprising the steps of:

- 20
- mixing a recombinant polypeptide as defined in claim 20 to 22 with sphingosine, labelled ATP and a candidate molecule of interest; and
 - measuring the level of conversion of sphingosine to labelled sphingosine-1-phosphate (S1P).

25 28) A kit for the screening of candidate molecules which are inhibitors of hSK, the said kit comprising:

- a recombinant polypeptide as defined in claim 20 to 22 ; and, optionally,
- labelled ATP and sphingosine.

GTCCCGGGAATTAGTCGGGCGCTCCACCTCTGGCAGCTGCGGCCCGGACTCCGCCAGC- 60
GCTGTCCTCTCCTCAGGTCAGCCGCGCAGGGAATGACACCGGTGCTCCTACAGCC- 120
ACGGCTCCGGCGGGAAGGAGCCCAAGCGGCCCTGCGACGCGCCCGCTGGGCAGGACC- 180
GATAAGGAAGGAGGAGCGCCGCGCAGGCGCCCAAGCGCCAGGAGCC- 240
CCTGGCAGCGGAGCCGCGGTCGAGGTTATGGATCCAGCGGCGGCCCGCGGCGTGC- 300
M D P A G G P R G V L- 11

TCCCGCGGCCCTGCGCGCTGCTGCTGCTGAACCCGCGCGCGCAAGGCAAGGCCT- 360
P R P C R V L V L L N P R G G K A L- 31

TGCAGCTCTTCCGGAGTCACGTGCAGCCCTTTTGGCTGAGGCTGAAATCTCCTTACAGC- 420
Q L F R S H V Q P L L A E A E I S F T L- 51

TGATGCTCACTGAGCGCGGGAACCAAGCGCGGAGCTGGTGCAGGAGCTGGGCC- 480
M L T E R R N H A R E L V R S E E L G R- 71

GCTGGGACGCTCTGGTGGTCATGCTGGAGACGGGCTGATGCACGAGGTGGTGAACGGGC- 540
W D A L V V M S G D G L M H E V V N G L- 91

Fig.1

TCATGGAGCGGCTGACTGGGAGACCGCCATCCAGAAAGCCCTGTGTAGCCTCCACGAG- 600
M E R P D W E T A I Q K P L C S L P A G- 111

GCTCTGGCAACGCGCTGGCAGCTTCCTTGAACCATATGCTGGCTATGAGCAGGTCACCA- 660
S G N A L A A S L N H Y A G Y E Q V T N- 131

ATGAAGACCTCCTGACCAACTGCACGCTATTGCTGTGCCCGCGCTGTGTACCCCATGA- 720
E D L L T N C T L L L C R R L L S P M N- 151

ACCTGCTGTCTCTGCACACGGCTTCGGGGCTGCGCCTCTCTCTGTGCTCAGCCTGGCCT- 780
L L S L H T A S G L R L F S V L S L A W- 171

GGGGCTTCATTGCTGATGTGGACCTAGAGAGTGAGAAAGTATCGGCGTCTGGGGGAGATGC- 840
G F I A D V D L E S E K Y R R L G E M R- 191

Fig. 1 (CONTINUED)

GCTTCACTCTGGGCACCTTCCTGCGTCTGGCAGCCCTGCGCACCTACCGCGGCCGACTGG- 900
F T L G T F L R L A A L R T Y R G R L A- 211
CCTACCTCCCTGTAGGAAGAGTGGGTTCCAAAGACACCTGCCCTCCCCCGTTGTGGTCCAGC- 960
Y L P V G R V G S K T P A S P V V Q Q- 231
AGGGCCCGGTAGATGCACACCTTGTGCCCACTGGAGGAGCCAGTGCCCTCTCACTGGACAG-1020
G P V D A H L V P L E E P V P S H W T V- 251
TGGTGCCCGACGAGGACTTGTGCTAGTCCCTGGCACTGCTGCACTCGCACCTGGGCAGTG-1080
V P D E D F V L V L A L L H S H L G S E- 271
AGATGTTGTGTCACCCATGGGCCCGCTGTGCAGCTGGCGTCATGCATCTGTTCTACGTGC-1140
M F A A P M G R C A A G V M H L F Y V R- 291

Fig. 1 (CONTINUED)

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GGCGGGAGTGCTCTCGTGCCATGCTGCTGCGCCTCTTCCCTGGCCATGGAAGGCGAGGC-1200
A G V S R A M L L R L F L A M E K G R H- 311
ATATGGAGTAAATGCCCCCTACTTGGTATATGTGCCCGTGTCGCCCTTCCGCTTGGAGC-1260
M E Y E C P Y L V Y V P V V A F R L E P- 331
CCAAGGATGGGAAGGTGTGTTGTCAGTGGGGAATTGATGGTTAGCGAGGCCGTGC-1320
K D G K G V F A V D G E L M V S E A V Q 351
AGGCCAGGTGCACCCCAACTACTTCTGGATGGTCAGCGGTTGCGTGAGCCCCCGCCCA-1380
G Q V H P N Y F W M V S G C V E P P S- 371
GCTGGAAGCCCCAGCAGATGCCACCGCCAGAAAGAGCCCTTATGACCCCTGGGCGCGTTG-1440
W K P Q Q M P P P E E P L * 384
TGCCCTTAGTGCTACTTGCAGGACCCCTTCCCTCCCTAGGGCTGCAGGGCCCTGTCCAC-1500
AGCTCCTGTGGGGGTGGAGGAGACTCCTCTGGAGAGGGTGAGAGGTGAGGCTATGCT-1560
TTGGGGGACAGGCCAGAAATGAAGTCCCTGGGTCAGGAGCCAGCTGGCTGGGCCCCAGCTG-1620
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Fig. 1 (CONTINUED)

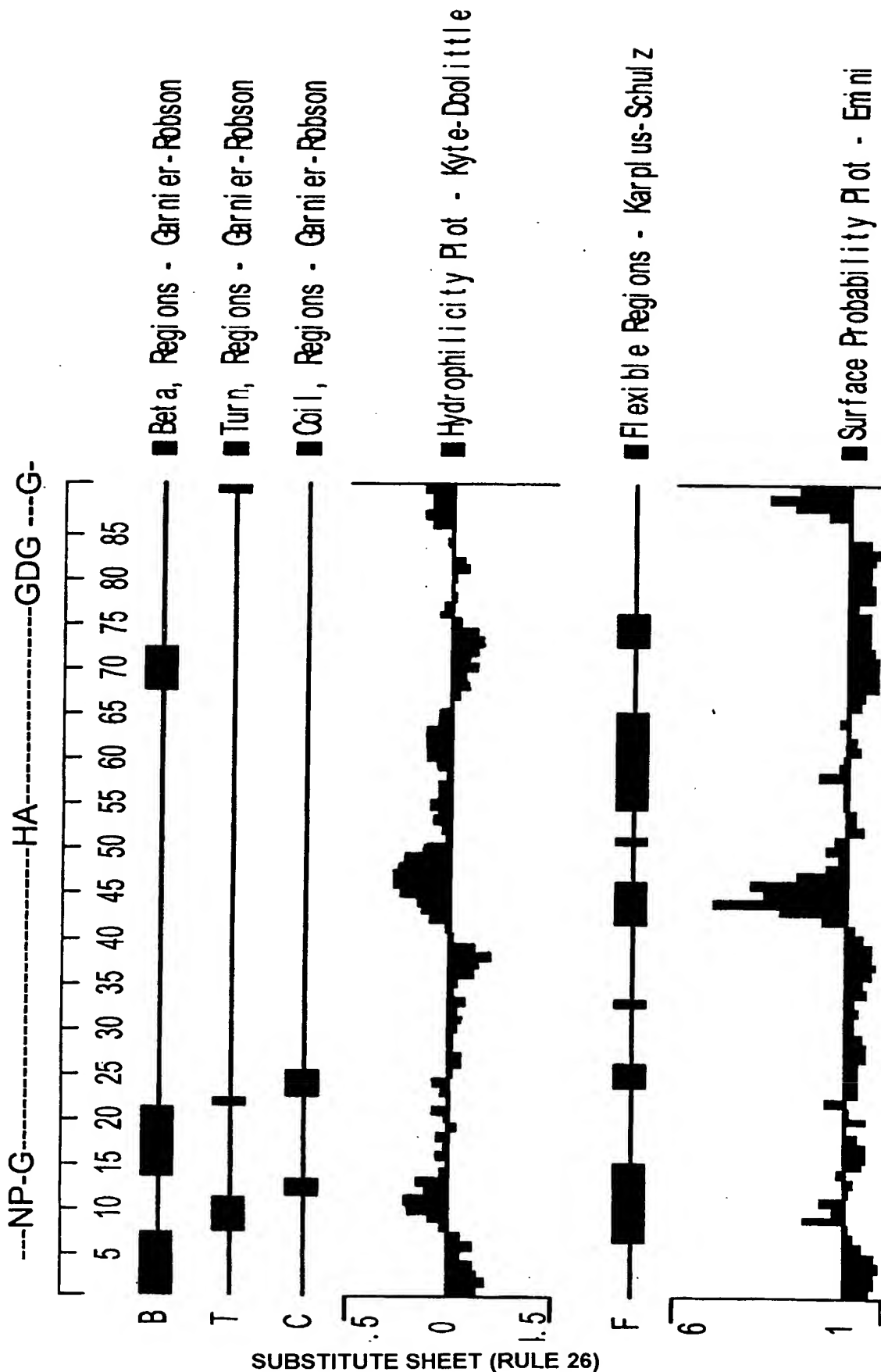


Fig. 2A

```

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-----RLLVFVNPFGGKKSAREIFVKEVKPL--FEDAD CAA18718_A_t_hypoth.PRO
-----RFIVFINPHGGKGKAKHIWSEAEVP--FSSAH CAB11477_S_p_hypoth.PRO
-----SIFVINPFPGGKGKAKKLFMTKAKPL--LLASR S51398_S_c_hypoth.PRO
-----SILVINPHGGKGKTAKNLFELTKARPI--LVE[SG S67059_S_c_hypoth.PRO
)EQCRGNLLVFINPNSGTGKSLETFANTVGP[--LDKSL CAA91259_C_e_hypoth.PRO
-----NIIIFINPFNGGNGKAQKIFKDNVDAFFWLT[PG[ AAC67466_C_e.PRO
-----RVLVLLNPFQGGKGKALQLFQSRVQPF--LEEAE Mouse SPHK 1a.pro
-----RVLVLLNPRGGKGKALQLFRSHVQPI--LAEAE huSPHK.PRO
16.....
-IS-EVVLTERPNHARD--LAKSEDLS--KYDGLAVASGDGLMHHEVVN
-----EH-RSFLTEHPGHA[EV--LARQISTIQEYK[LRKRLIVIGGDGTMHEVVN CAB14972_B_s.PRO
-VQLEIQETKYQLHAK[EE--FVKSM[DV[---KYDGI[VCVSGDGILVE[V CAA18718_A_t_hypoth.PRO
-SICEVVLTRRKDHAKS--IAKNLDVG--SYDGILSVGGDGLFHEVI CAB11477_S_p_hypoth.PRO
-C[IEVVTYTKY[PGHAIE--IAREMDID--KYDTIACASGDGIPHEVIN S51398_S_c_hypoth.PRO
-CKIEIAYTKYARHAID--IAKDLDIS--KYDTIACASGDGIPYEVIN S67059_S_c_hypoth.PRO
IRY-EVVVTTGPNHARN--V[MTKADLG--KFNGLVILSGDGLVFEALN CAA91259_C_e_hypoth.PRO
-RY-KVVLTERANHARDYI VEMPPEQWS--AIDGLVSVGGDGLFNE[LLS AAC67466_C_e.PRO
-TTFKLILTERKNHAR--ELVCAEEL--GHWDALAVMSGDGLMHHEVVN Mouse SPHK 1a.pro
-ISFTLMLTERRNHAR--ELVRSEEL--GRWDALVMSGDGLMHHEVVN huSPHK.PRO
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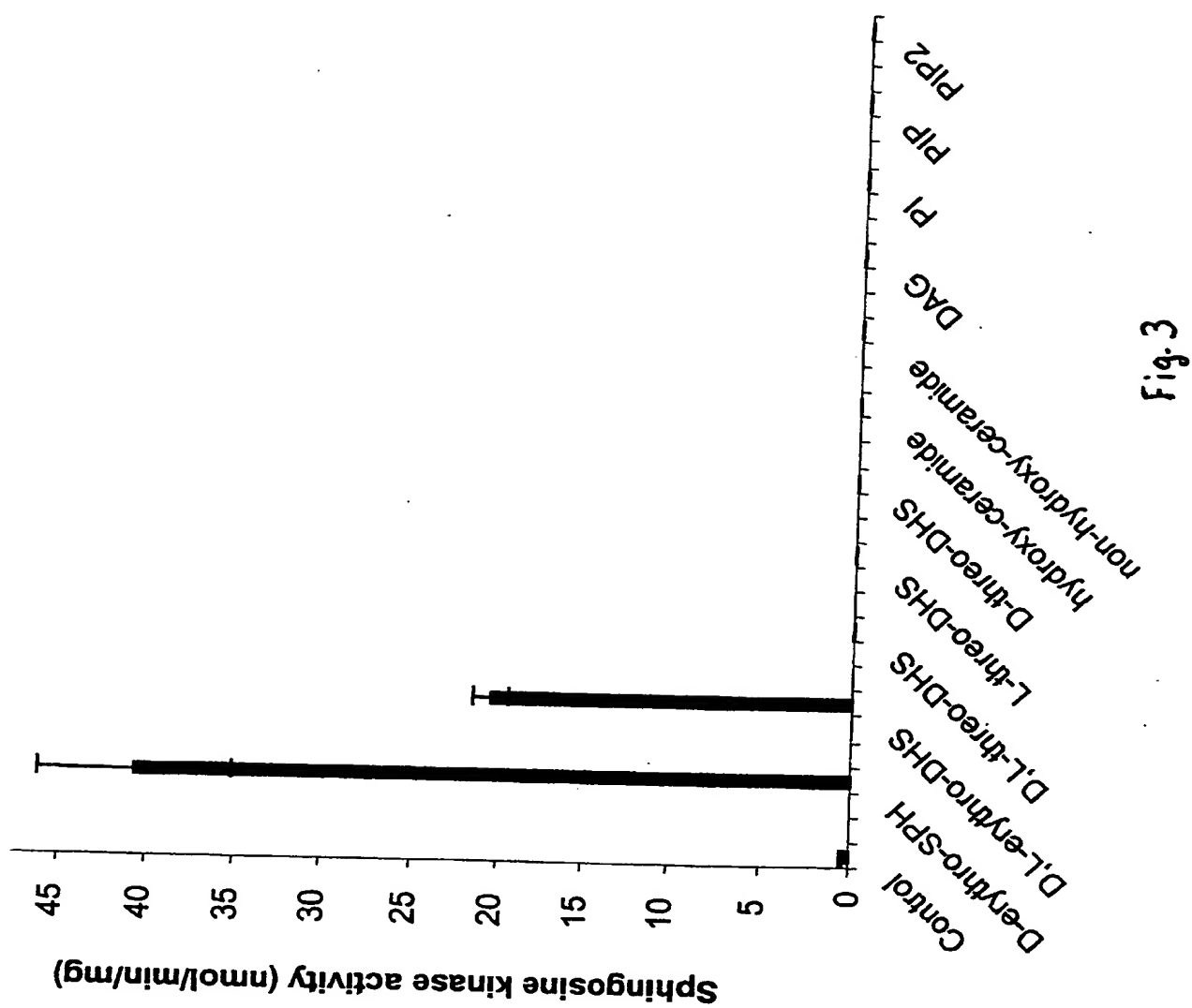
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Fig. 2B

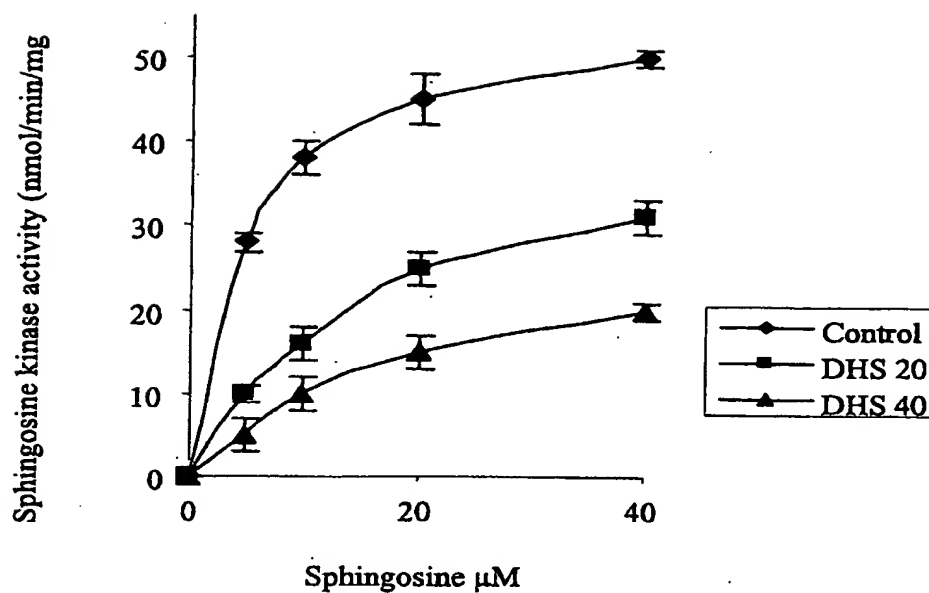
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NGLGERDDYLEAFKLPV	CAB11477_S_p_hypoth.PRO
GLYQRPD	S51398_S_c_hypoth.PRO
GLYRRPD	S67059_S_c_hypoth.PRO
GLLCRED	CAA91259_C_e_hypoth.PRO
GALLRTQ	AAC67466_C_e.PRO
GLMERP	Mouse SPHK 1a.pro
GLMERP	huSPHK.PRO

..... 95

Fig. 2B (CONTINUED)



A1



A2

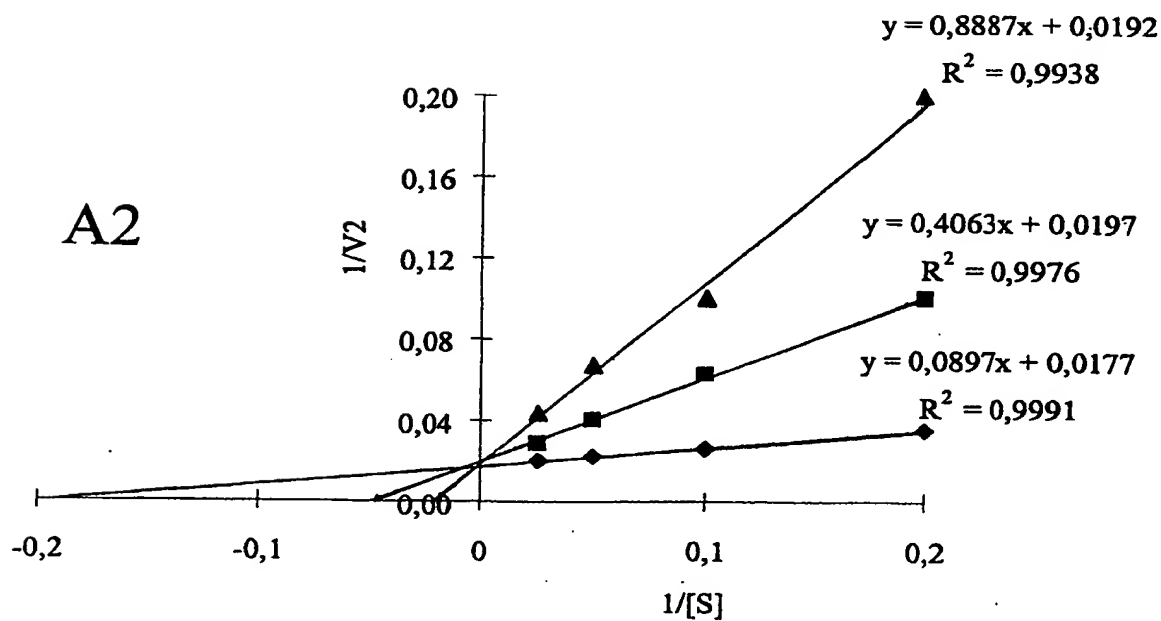


Fig. 4A

SUBSTITUTE SHEET (RULE 26)

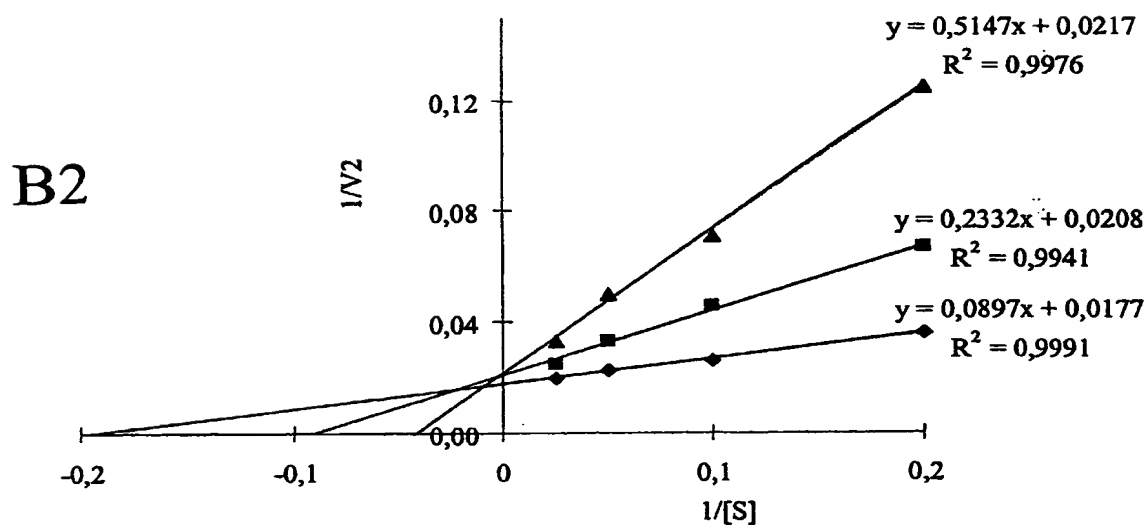
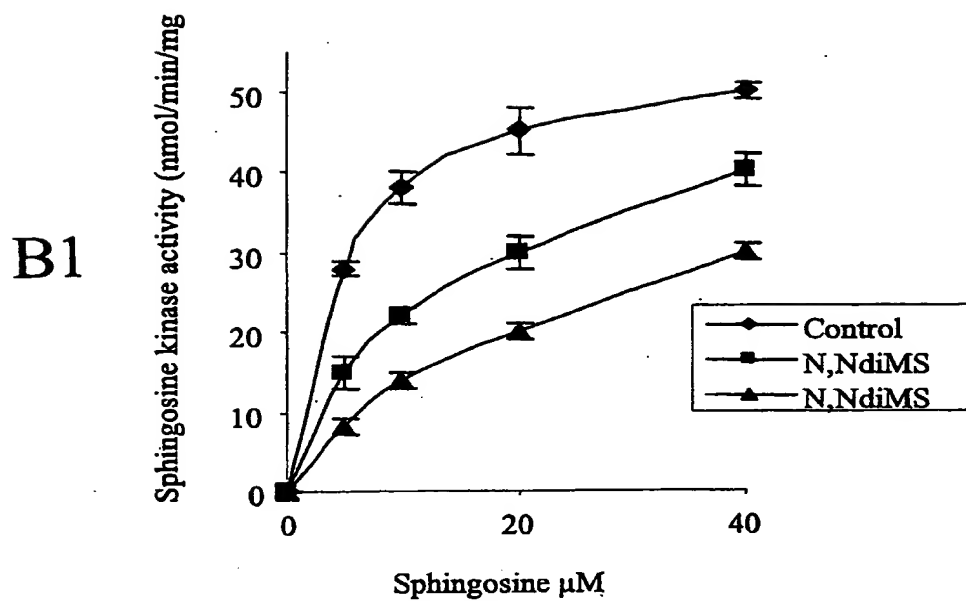


Fig. 4B

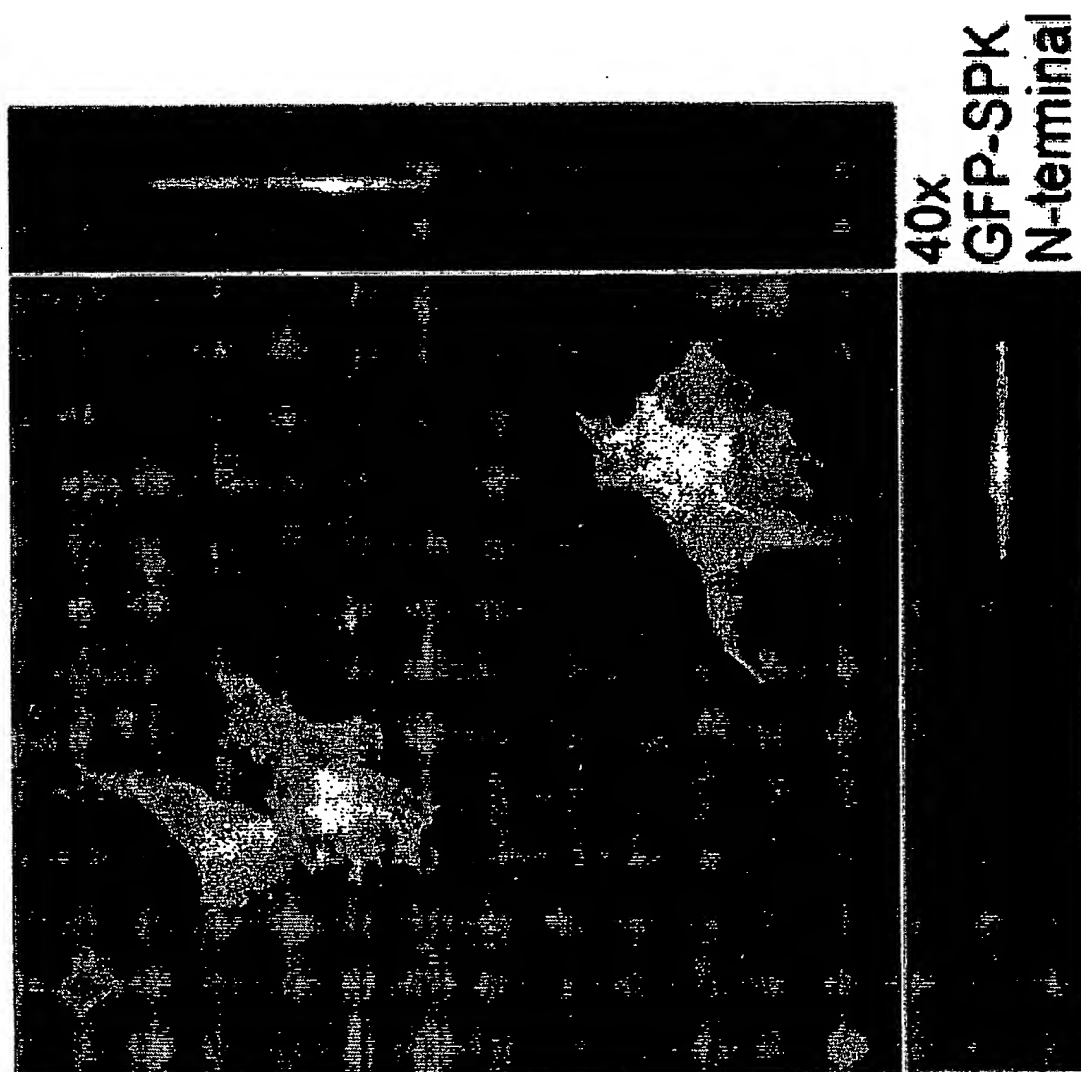


Fig. 5A

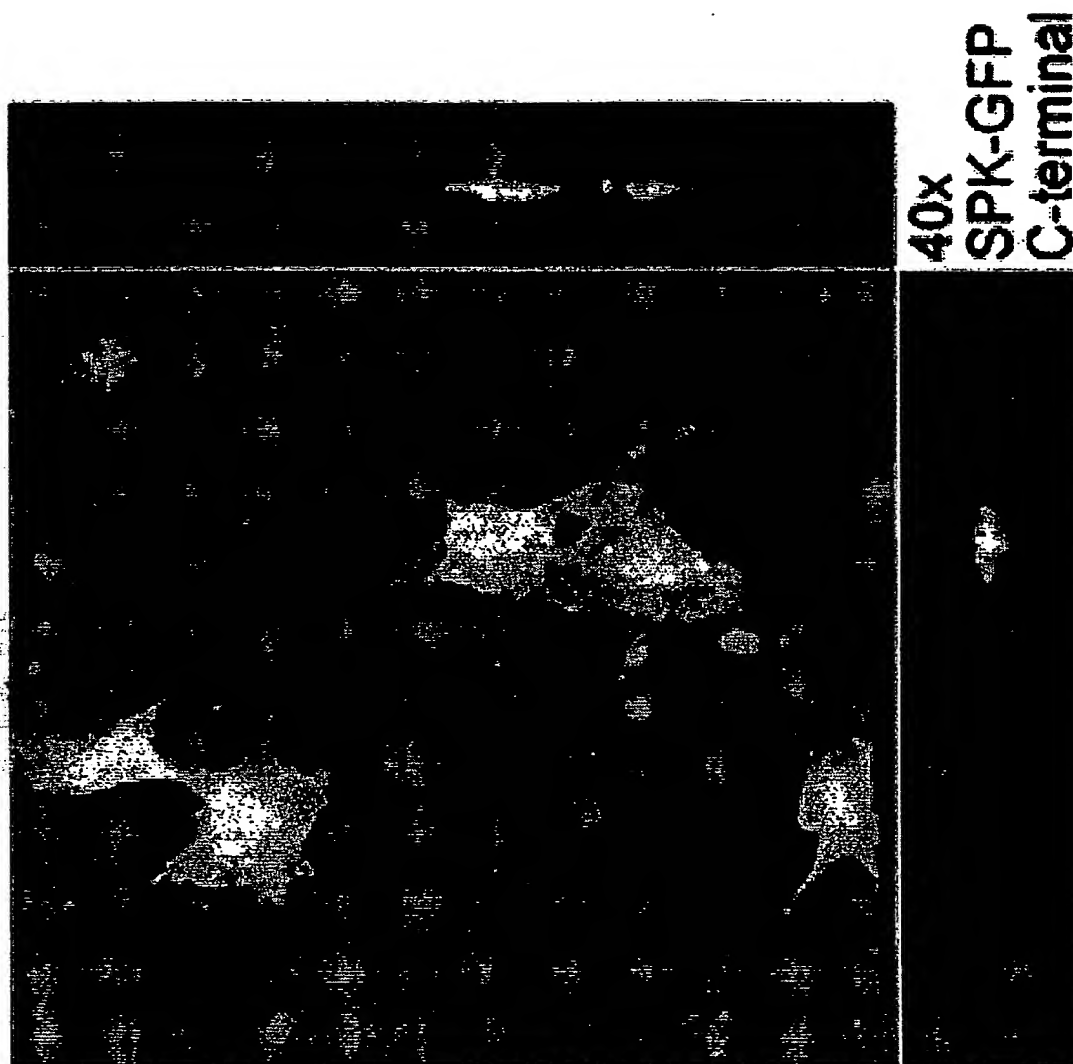


Fig. 5B

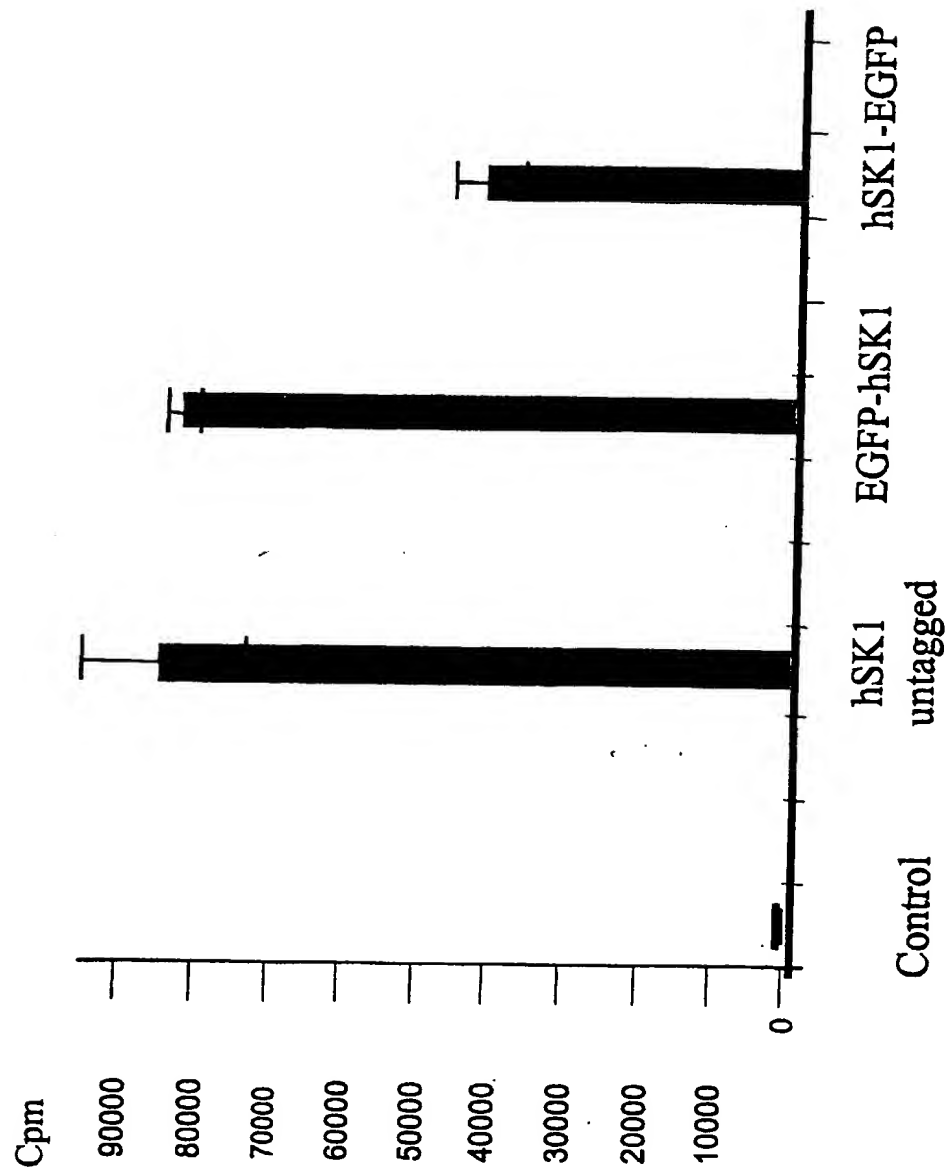


Fig. 6

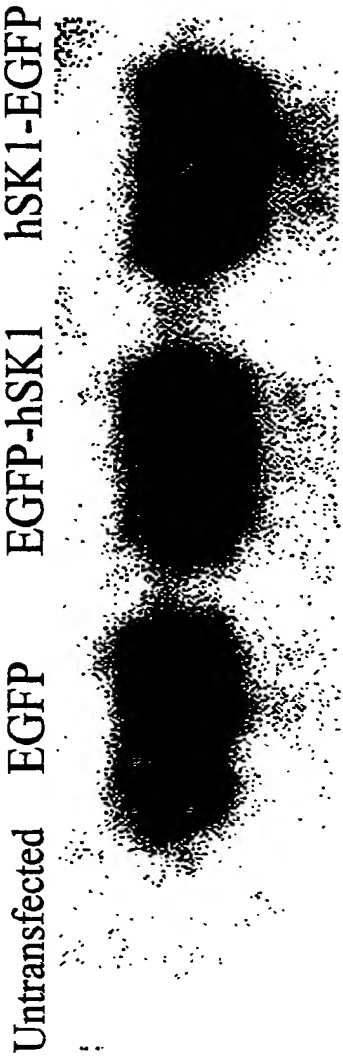


Fig. 7

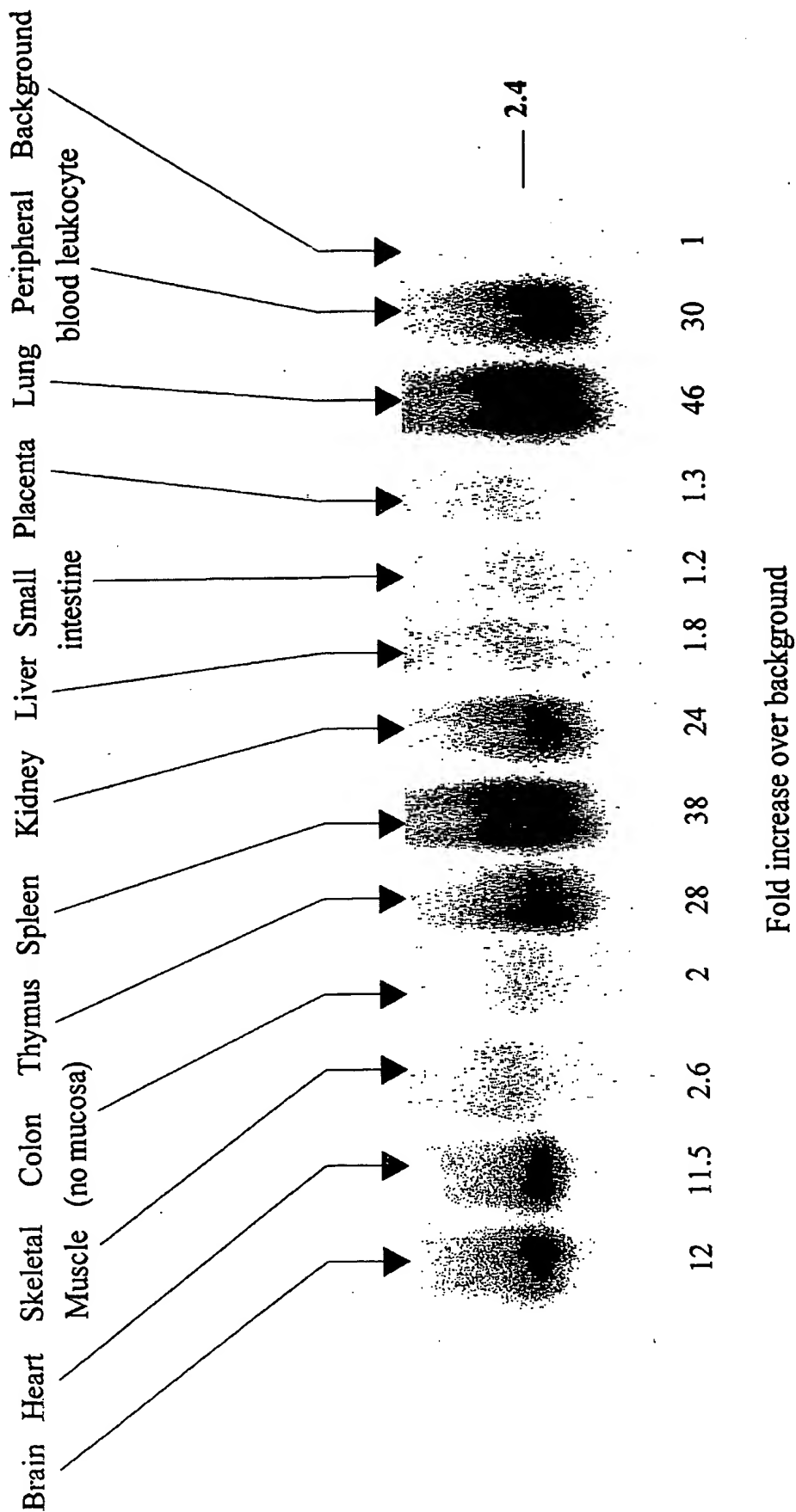


Fig. 8

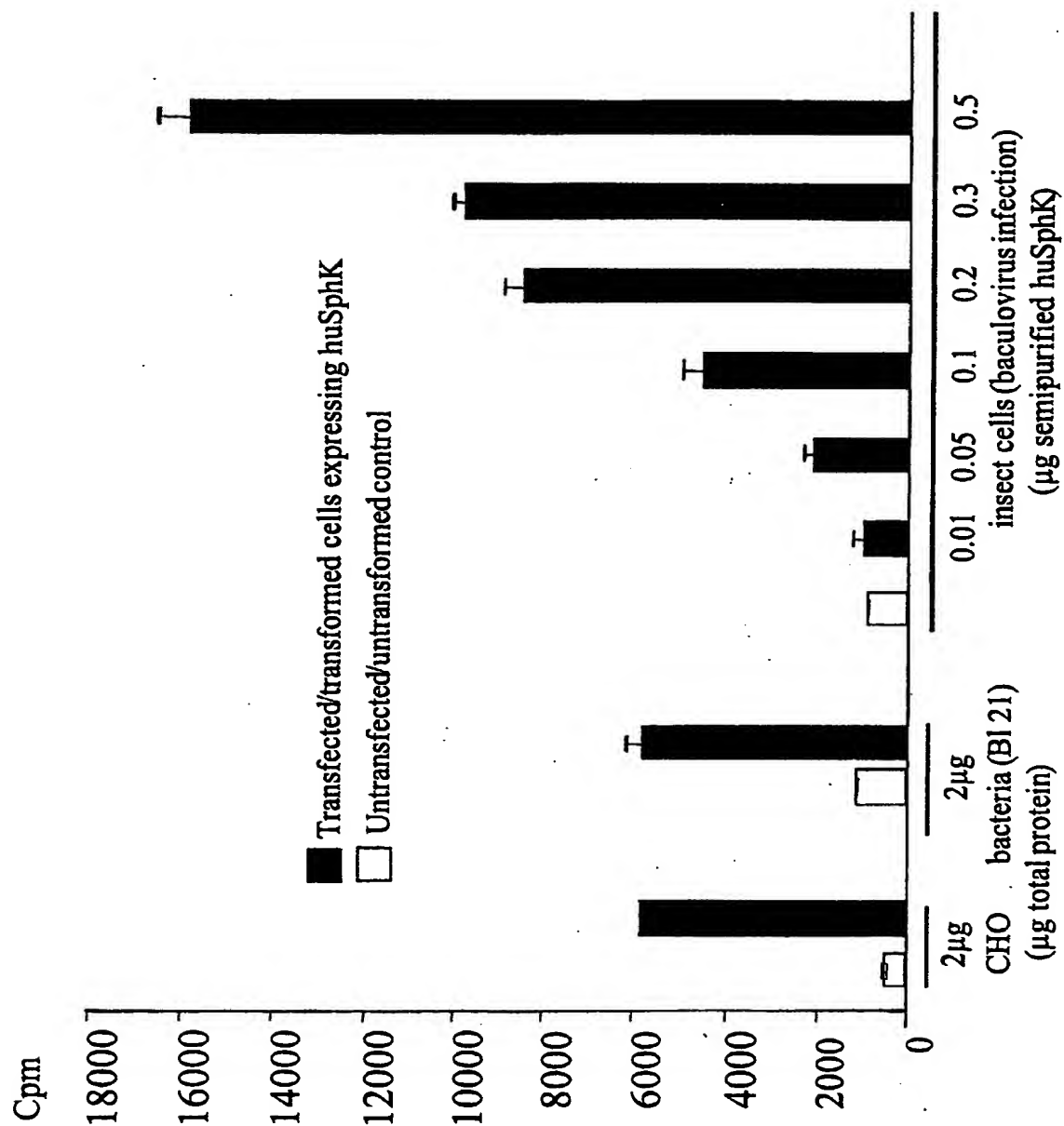


Fig. 9

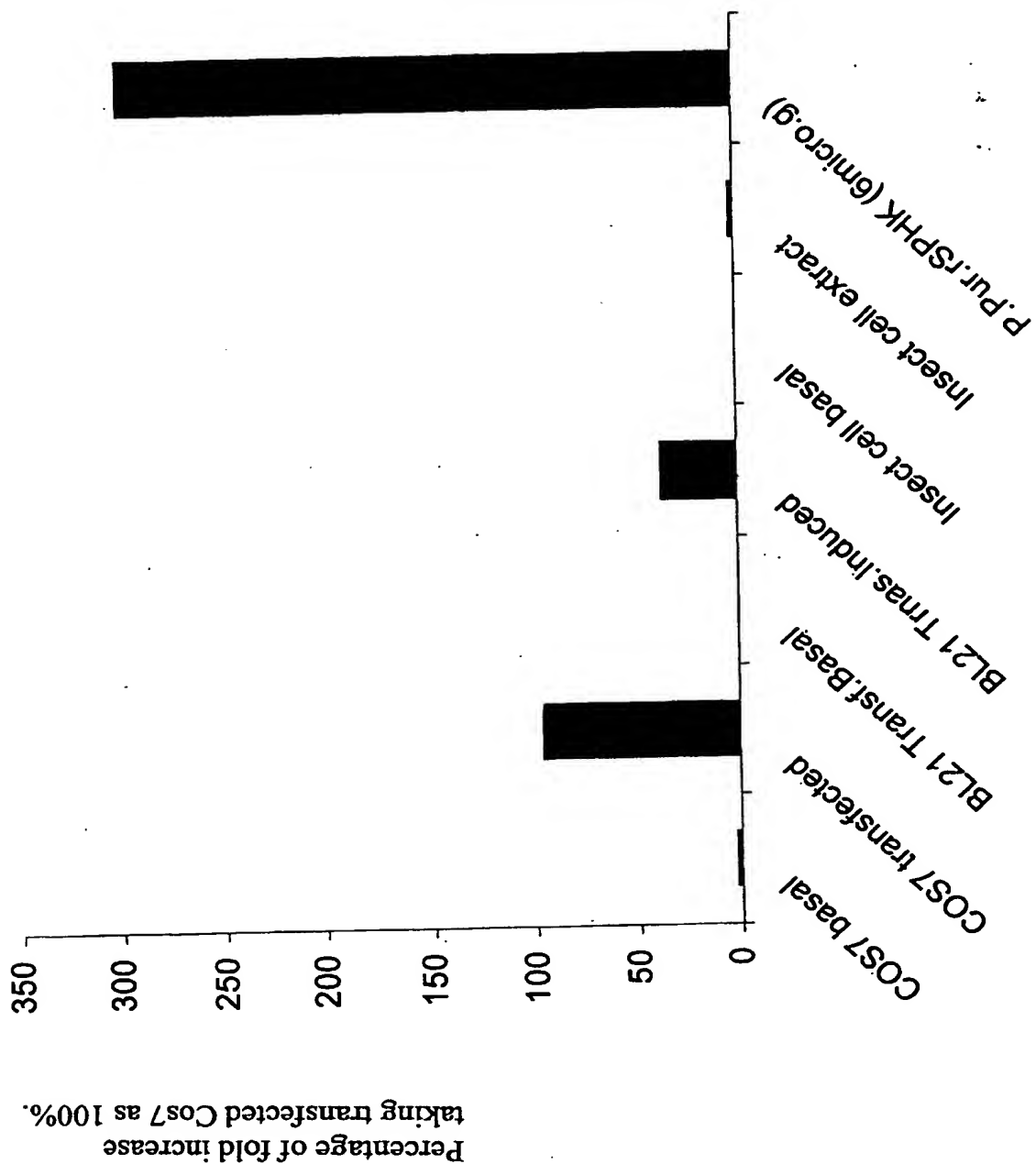


Fig. 10

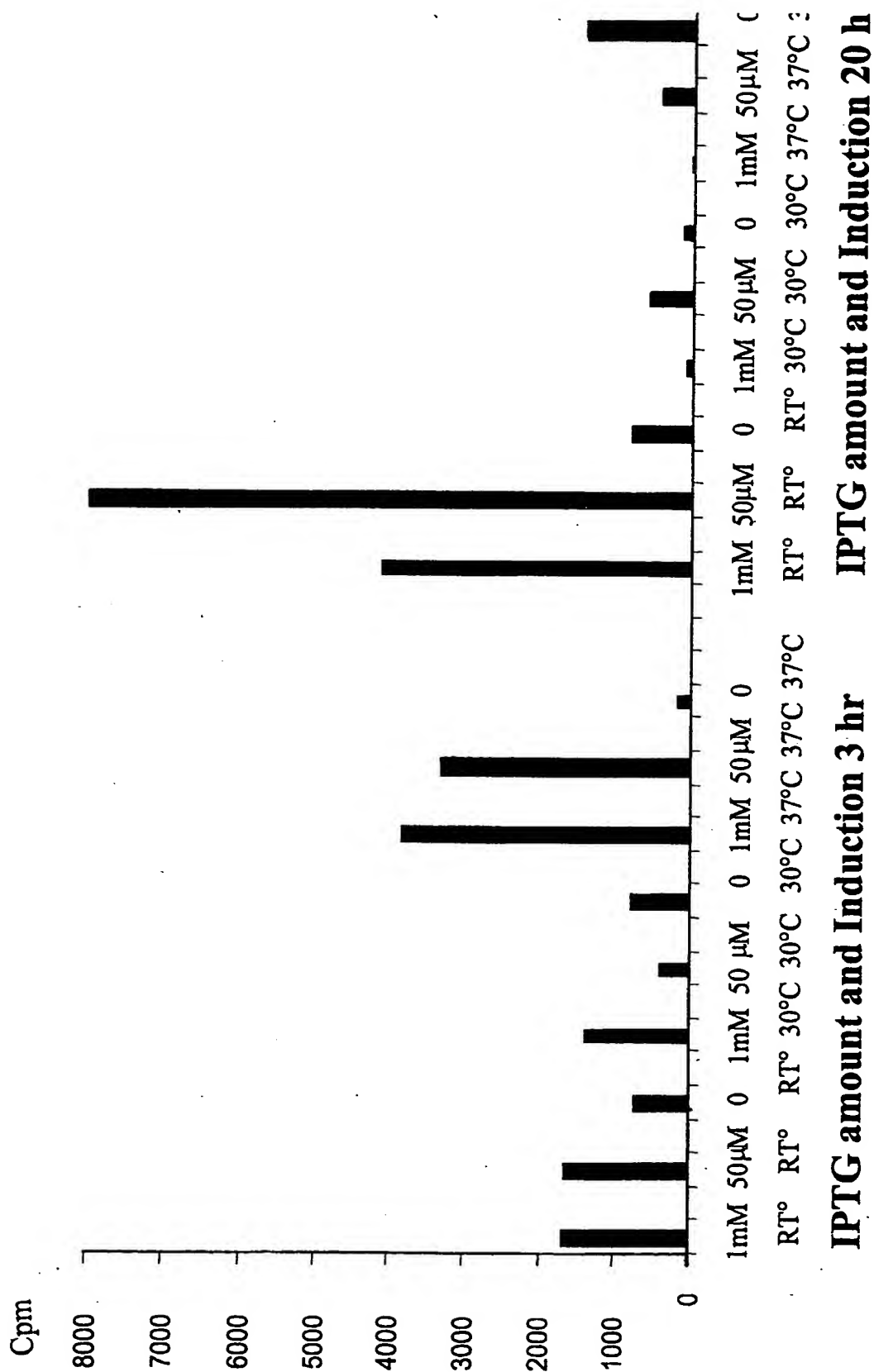


Fig. 44

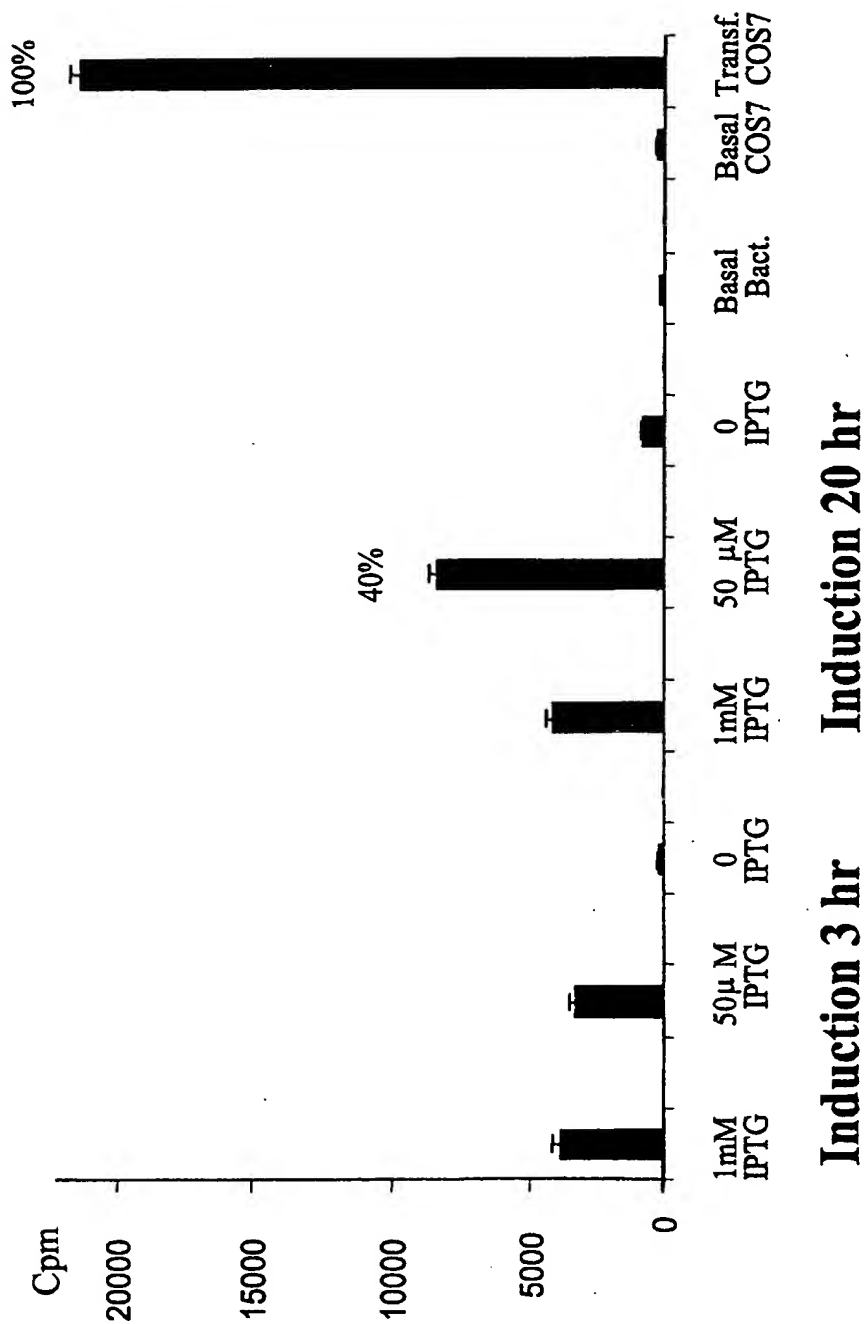


Fig. 12

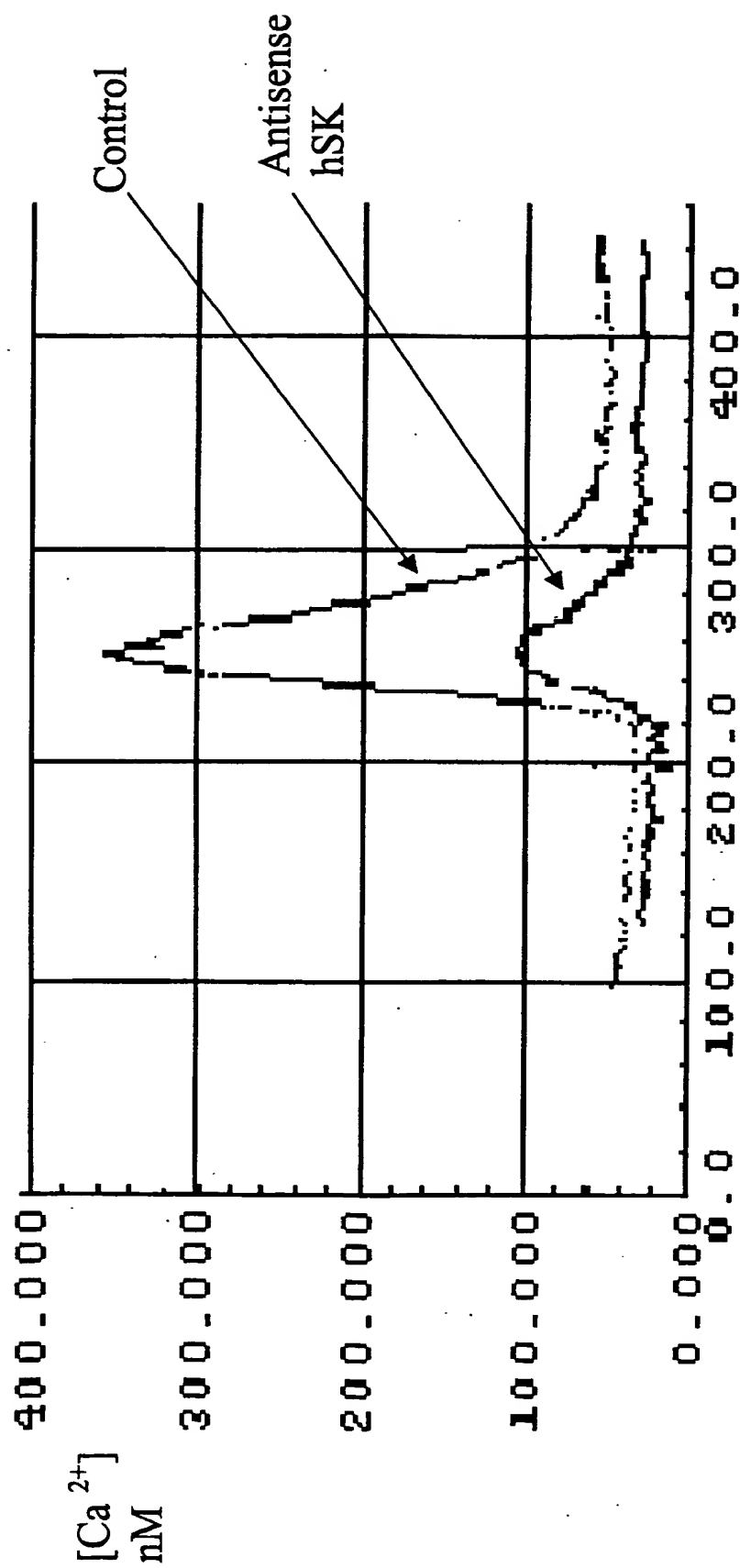


Fig. 13

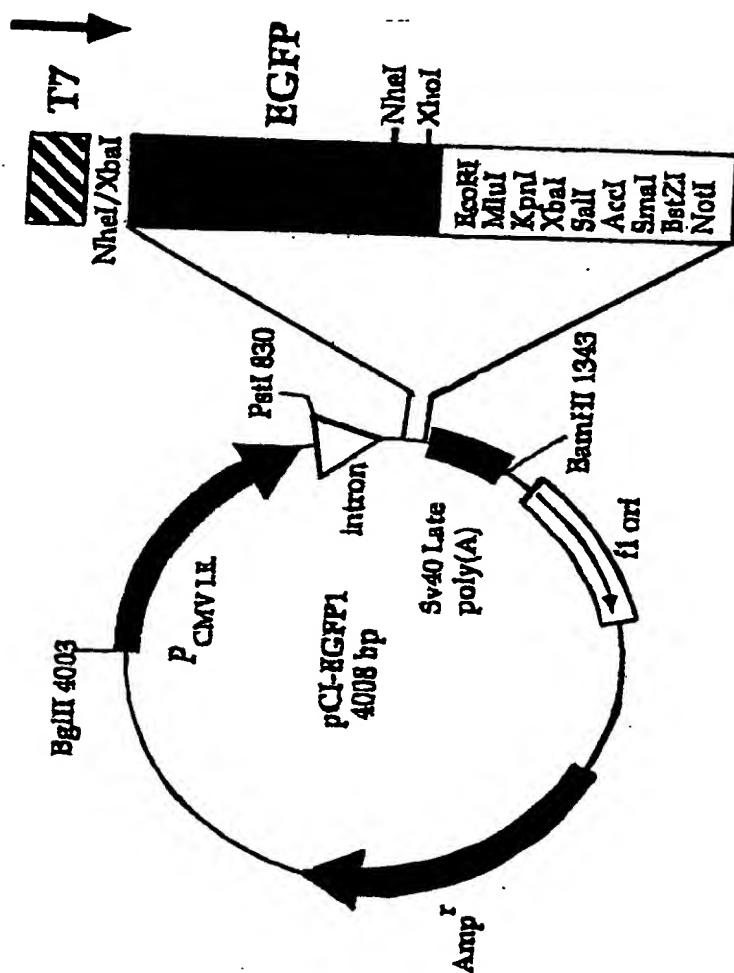


Fig. 14

--- GCT AGC CTC GAG XXX XXX ...
 ↑ NheI
 ↑ XbaI

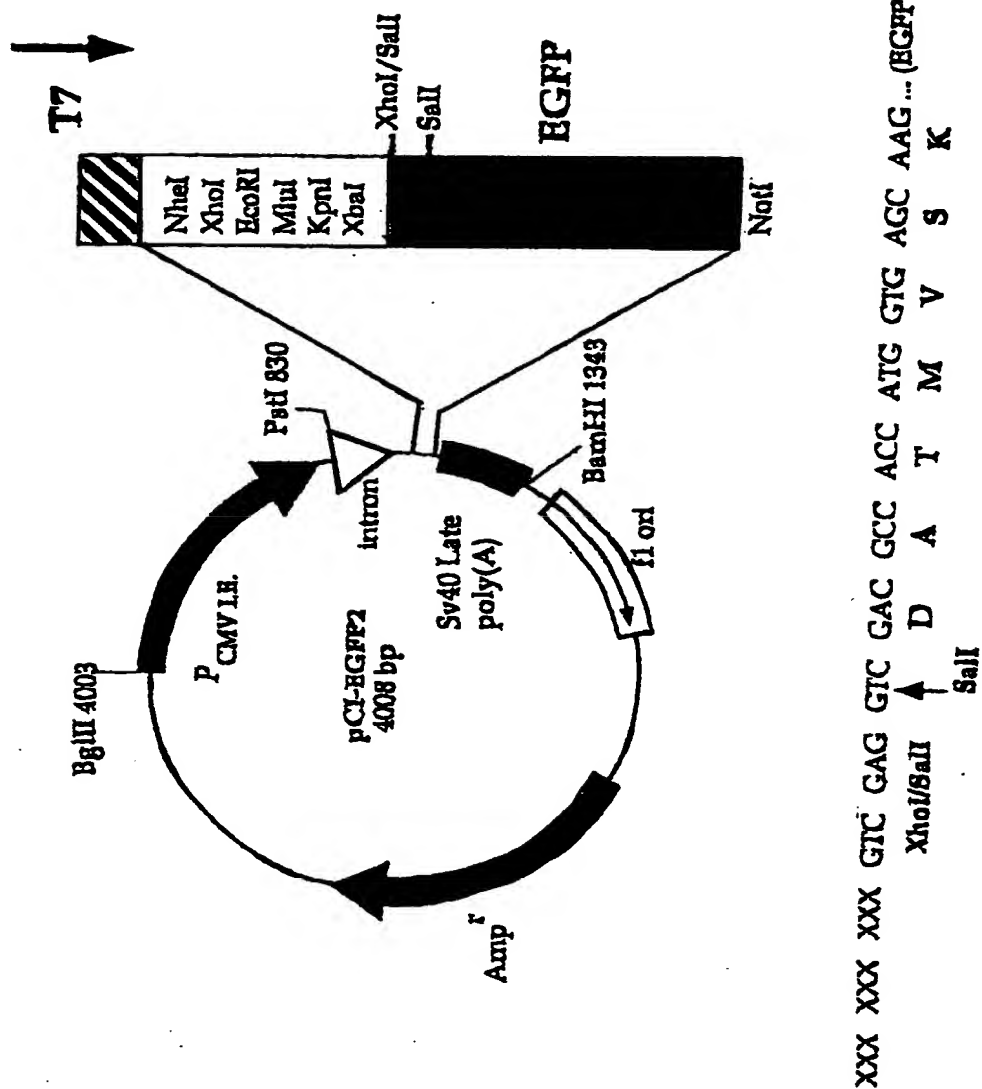


Fig. 15

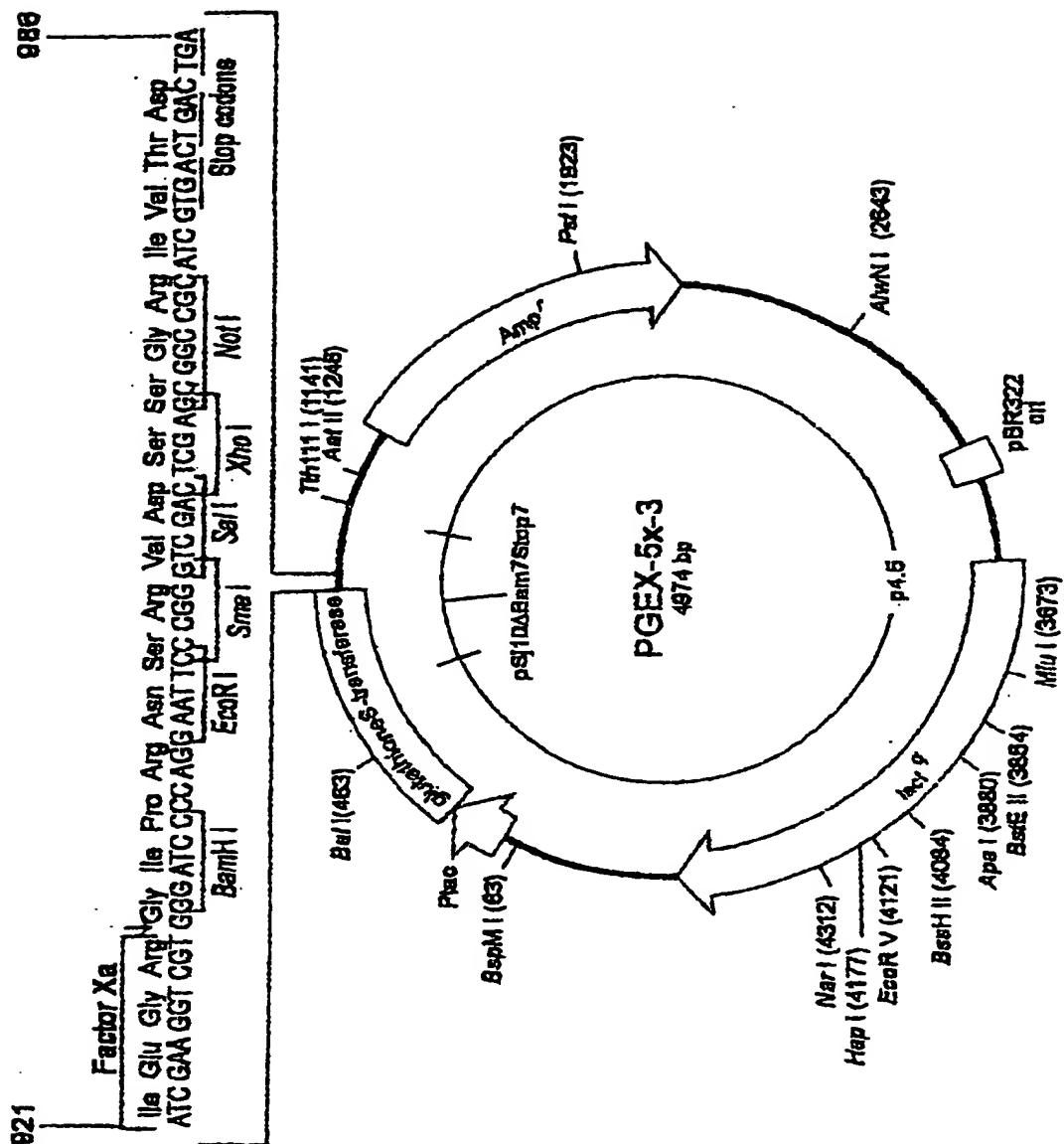


FIG. 16

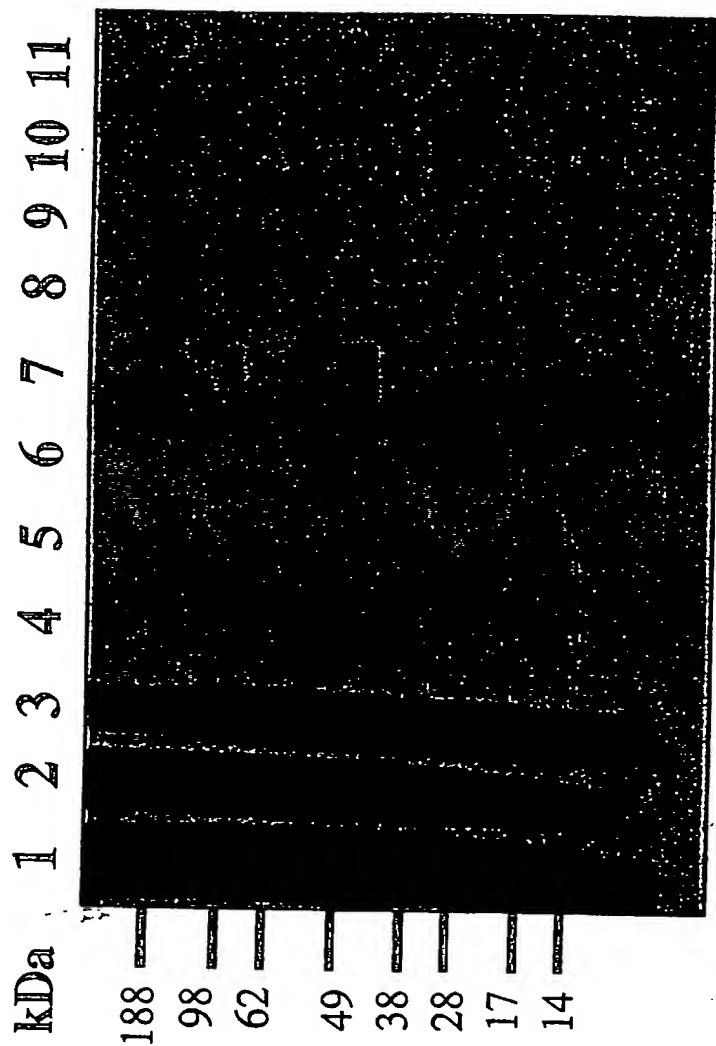


FIG. 17

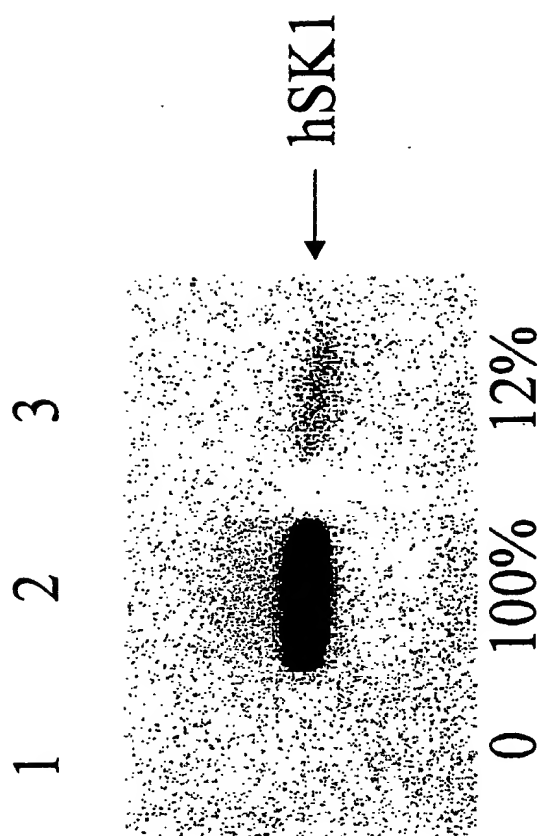


FIG. 18

SEQUENCE LISTING

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<141>

<160> 24

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<211> 1719

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 20 25 30
 30 Leu Phe Arg Ser His Val Gln Pro Leu Leu Ala Glu Ala Glu Ile Ser
 35 40 45
 Phe Thr Leu Met Leu Thr Glu Arg Arg Asn His Ala Arg Glu Leu Val
 50 55 60
 35 Arg Ser Glu Glu Leu Gly Arg Trp Asp Ala Leu Val Val Met Ser Gly
 65 70 75 80
 40 Asp Gly Leu Met His Glu Val Val Asn Gly Leu Met Glu Arg Pro Asp
 85 90 95
 Trp Glu Thr Ala Ile Gln Lys Pro Leu Cys Ser Leu Pro Ala Gly Ser
 100 105 110
 45 Gly Asn Ala Leu Ala Ala Ser Leu Asn His Tyr Ala Gly Tyr Glu Gln
 115 120 125
 Val Thr Asn Glu Asp Leu Leu Thr Asn Cys Thr Leu Leu Leu Cys Arg
 130 135 140
 50 Arg Leu Leu Ser Pro Met Asn Leu Leu Ser Leu His Thr Ala Ser Gly
 145 150 155 160
 55 Leu Arg Leu Phe Ser Val Leu Ser Leu Ala Trp Gly Phe Ile Ala Asp
 165 170 175

Val Asp Leu Glu Ser Glu Lys Tyr Arg Arg Leu Gly Glu Met Arg Phe
 180 185 190
 5 Thr Leu Gly Thr Phe Leu Arg Leu Ala Ala Leu Arg Thr Tyr Arg Gly
 195 200 205
 Arg Leu Ala Tyr Leu Pro Val Gly Arg Val Gly Ser Lys Thr Pro Ala
 210 215 220
 10 Ser Pro Val Val Val Gln Gln Gly Pro Val Asp Ala His Leu Val Pro
 225 230 235 240
 Leu Glu Glu Pro Val Pro Ser His Trp Thr Val Val Pro Asp Glu Asp
 15 245 250 255
 Phe Val Leu Val Leu Ala Leu Leu His Ser His Leu Gly Ser Glu Met
 260 265 270
 20 Phe Ala Ala Pro Met Gly Arg Cys Ala Ala Gly Val Met His Leu Phe
 275 280 285
 Tyr Val Arg Ala Gly Val Ser Arg Ala Met Leu Leu Arg Leu Phe Leu
 290 295 300
 25 Ala Met Glu Lys Gly Arg His Met Glu Tyr Glu Cys Pro Tyr Leu Val
 305 310 315 320
 Tyr Val Pro Val Val Ala Phe Arg Leu Glu Pro Lys Asp Gly Lys Gly
 30 325 330 335
 Val Phe Ala Val Asp Gly Glu Leu Met Val Ser Glu Ala Val Gln Gly
 340 345 350
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20 25 30
Ser Phe Thr Leu Met Leu Thr Glu Arg Arg Asn His Ala Arg Glu Leu
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1 5 10 15

Arg Phe Thr Leu
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1 5 10 15

Pro Pro Pro Glu Glu
20 20

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120
cggcggaacc acgcgcggga gctggtgagg tcggaggagc tgggccgctg ggacgctctg
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50

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